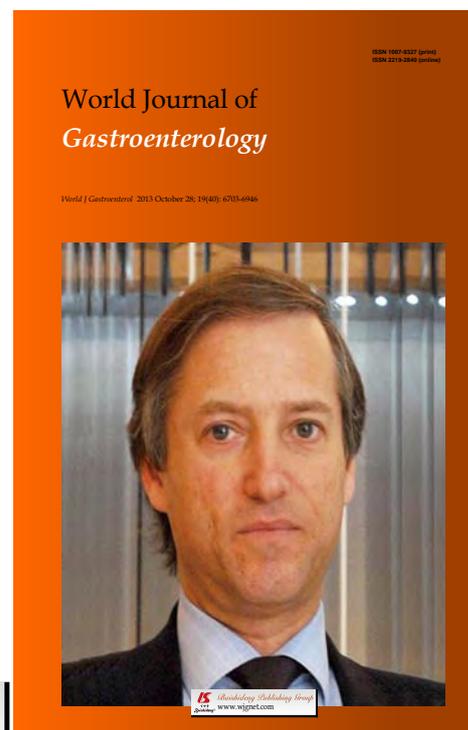


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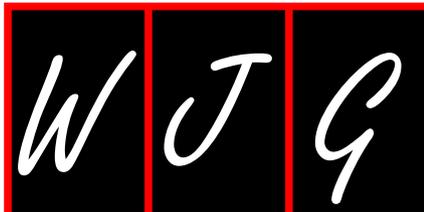
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## HLA class II associated with outcomes of hepatitis B and C infections

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### Abstract

Several factors influence the clinical course of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. The human leukocyte antigen (HLA) system, the major histocompatibility complex (MHC) in humans, has been considered one of the most important host factors with respect to outcomes. To date, conventional genotyping studies have shown that *HLA* class II loci are mainly associated with spontaneous clearance of HBV and HCV. However, the specific HLA locus associated with the outcomes of hepatitis virus infection remains unclear. A recent genome-wide association study (GWAS) using a comprehensive approach for human genotyping demonstrated single nucleotide polymorphisms (SNPs) associated with the outcomes of hepatitis virus infection. Examination of large numbers of cohorts revealed that several SNPs in both *HLA-DPA1* and *HLA-DPB1* loci are associated with persistent HBV infection in Asian populations. To date, however, few studies have focused on *HLA-DP* because polymorphisms of *HLA-DP* haplotype do not vary greatly as compared with other loci of *HLA*. There are not enough studies to reveal the function of *HLA-DP*. GWAS additionally detected candidate SNPs within HLA loci associated with chronic HBV or HCV hepatitis, hepatic fibrosis, and the development of hepatocellular carcinoma. The results

of one cohort were not always consistent with those of other cohorts. To solve several controversial issues, it is necessary to validate reported SNPs on *HLA* loci in global populations and to elucidate the *HLA*-allele-regulated molecular response to hepatitis virus infection.

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**Key words:** Hepatitis B virus; Hepatitis C virus; Hepatocarcinogenesis; Human leukocyte antigen; Genome-wide association studies; Genotyping; Persistent infection

**Core tip:** Conventional genotyping studies have shown that human leukocyte antigen (*HLA*) typing was one of the most important host factors with respect to outcomes of hepatitis B and C virus infections. However, the specific HLA locus associated with the outcomes remains unclear. Recently a genome-wide association study for human genotyping demonstrated single nucleotide polymorphisms associated with the outcomes of hepatitis virus infection. Now it has been confirmed that several single nucleotide polymorphisms in both *HLA-DP* loci were associated with persistent hepatitis B virus infection in Asian populations.

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### INTRODUCTION

The human leukocyte antigen (HLA) system, the major histocompatibility complex (MHC) in humans, has long been considered the most important region in the human genome with respect to infection, inflammation, autoimmunity, and transplantation medicine<sup>[1,2]</sup>. In humans, *HLA* complex consists of more than 200 genes located

close together on chromosome 6. Genes in this complex are categorized into three basic groups: class I (*HLA-A*, *-B*, and *-C*), class II (*HLA-DR*, *-DQ*, and *-DP*), and class III (some genes involved in inflammation and other immune-system activities). Interactions among HLA-restricted T lymphocytes, B lymphocytes, natural killer (NK) cells, and cytokines influence immune response to viral infection. *HLA* class I and II molecules are expressed as cell surface antigens that bind to peptide epitopes on CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells, respectively. Effective presentation of viral antigens by the HLA system induces good immune response.

It is well known that some patients infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) spontaneously recover and can escape from persistent infection<sup>[3-5]</sup>. Progression of liver diseases by chronic viral infection also differs among patients. In addition, the response to HBV vaccination is different in each person. To identify immune systems against invaders in individual patients, *HLA* haplotypes related to persistent viral infection or providing protection against such infection have been examined. Singh *et al*<sup>[6]</sup> reported a detailed review about associations of *HLA* types with HBV and HCV infections among global populations. They speculated that there was a limited chance of detecting globally common *HLA* types related to outcomes or disease progression associated with hepatitis viral infection because *HLA* loci are diverse owing to racial admixture, environmental and selection pressure, and inherent polymorphic nature, leading to allelic variations among different ethnic groups.

Recent genome-wide association studies (GWAS) have demonstrated single nucleotide polymorphisms (SNPs) associated with the outcomes of hepatitis virus infection<sup>[7-14]</sup>. Imputation-based association analysis showed that some of the SNPs are located near *HLA* loci in chromosome 6p21<sup>[7,8,15]</sup>. Conventional genotyping and GWAS are different approaches for analysis. Conventional genotyping examines selected targeted genes, while GWAS can comprehensively examine hundreds of thousands of SNPs<sup>[16]</sup>. Although both approaches have suggested that *HLA* loci play important roles in the outcomes of viral hepatitis, the precise regions of *HLA* loci detected by each approach differed. In the present review, we summarize and compare the latest data obtained by GWAS with previous data obtained by conventional *HLA* typing.

## ASSOCIATION BETWEEN HEPATITIS VIRAL INFECTION AND *HLA* ALLELES IDENTIFIED BY GENOTYPING

Singh *et al*<sup>[6]</sup> suggested that an association of *HLA DR\*13* alleles in *HLA* Class II was protective in both HBV and HCV infections in several populations. *HLA DRB1\*11* and *HLA DQB1\*0301* were protective in HCV infection, but were associated with persistent HBV infection.

A recent meta-analysis showed that *HLA-DR\*03* and

*HLA-DR\*07* were associated with an increased risk of persistent HBV infection in 19 individual case-control studies including 9 Han Chinese cohorts, 3 Korean cohorts, 2 Iranian cohorts, and 1 cohort each of Caucasian, Gambian, Taiwanese, Thai, and Turkish subjects<sup>[17]</sup>. In contrast, *HLA-DR\*04* and *HLA-DR\*13* were associated with clearance of HBV infection. In Chinese Han populations, *HLA-DR\*01* was associated with clearance of HBV infection, while in other ethnic groups there was no association between *HLA-DR\*01* and HBV infection.

As for HCV infection, a study performed in patients from the United Kingdom and the United States reported that the inhibitory NK cell receptor KIR2DL3 and *HLA-C1* ligand, *HLA* class I interact directly to promote spontaneous viral clearance<sup>[18]</sup>. In global populations, *HLA* class II, especially several alleles in *HLA-DRB1*, has been linked to persistent HCV infection<sup>[19,20]</sup>. Interestingly, Spanish and American groups reported an association between *MICA* genotypes in *HLA* class III and clearance of HCV<sup>[21,22]</sup>.

## ASSOCIATION BETWEEN HEPATITIS VIRAL INFECTION AND SNPS IN *HLA* LOCUS IDENTIFIED BY GWAS

A recent GWAS discovered many SNP candidates associated with common diseases<sup>[16]</sup>. In research on viral hepatitis, several SNPs associated with outcomes, including the *HLA* coding region of chromosome 6p21.3, were detected by GWAS.

### *HBV* infection

Kamatani *et al*<sup>[7]</sup> reported the results of a case-control association study of HBV infection in 2009. They showed that rs3077 SNP near *HLA-DPA1* gene and rs9277535 SNP near *HLA-DPB1* were associated with persistent HBV infection in Japanese cohorts. In addition, *HLA* haplotype analysis showed that *HLA-DPA1\*0202-DPB1\*0501* and *HLA-DPA1\*0202-DPB1\*0301* were risk types for persistent HBV infection, and *HLA-DPA1\*0103-DPB1\*0402* and *HLA-DPA1\*0103-DPB1\*0401* were protective types for HBV infection. The same group performed a second GWAS analysis involving a larger number of cohorts<sup>[8]</sup>. The study validated that rs3077 SNP near *HLA-DPA1* gene and rs9277535 SNP near *HLA-DPB1* were strongly associated with persistent HBV infection. Other SNPs, rs2856718 and rs7453920 within the *HLA-DQ* locus, were also associated with persistent HBV infection. Moreover, HLA haplotype analysis indicated that *HLA-DQA1\*0102-DQB1\*0303* and *HLA-DQA1\*0301-DQB1\*0601* were risk types for persistent HBV infection, while *HLA-DQA1\*0102-DQB1\*0604* and *HLA-DQA1\*0101-DQB1\*0501* were protective types for HBV infection. GWAS of Han Chinese populations also showed that the *HLA-DPA1* and *HLA-DPB1* genes were related to persistent HBV infection. The first study from China indicated that 4 SNPs related to *HLA-*

**Table 1** Single nucleotide polymorphisms within human leukocyte antigen loci associated with outcomes of hepatitis B virus infection

Ethnic group	Outcome	HLA locus	SNP	Odds	95%CI	HLA haplotype	Odds	Ref.
Japanese	Chronic infection	HLA-DPA1	rs3077	0.56	0.51-0.61			[7]
		HLA-DPB1	rs9277535	0.57	0.52-0.62			
						DPA1*0202-DPB1*0501	1.45	
						DPA1*0202-DPB1*0301	2.31	
						DPA1*0103-DPB1*0402	0.52	
Japanese	Chronic infection	HLA-DQ	rs2856718	1.43	1.33-1.54			[8]
			rs7453920	1.66	1.49-1.85			
						DQA1*0102-DQB1*0303	19.3	
						DQA1*0301-DQB1*0601	5.02	
						DQA1*0102-DQB1*0604	0.16	
Chinese	Chronic infection	HLA-DPA1	rs2395309	0.71	0.59-0.86			[9]
		HLA-DPA1	rs3077	0.64	0.53-0.78			
		HLA-DPA1	rs2301220	0.67	0.56-0.81			
		HLA-DPA1	rs9277341	1.77	1.39-2.25			
		HLA-DPB1	rs3135021	0.78	0.64-0.94			
		HLA-DPB1	rs9277535	0.56	0.47-0.68			
		HLA-DPB1	rs10484569	1.60	1.33-1.93			
		HLA-DPB1	rs3128917	1.91	1.59-2.30			
		HLA-DPB1	rs2281388	1.66	1.38-2.01			
		HLA-DPB1	rs3117222	0.51	0.42-0.61			
Indonesian	Vaccine response	HLA-DR	rs3135363	1.59	1.45-1.73			[10]
		HLA-DPB1	rs9277535	0.82	0.71-0.96			
		HLA-III	rs9267665	2.13	1.82-2.49			
Chinese	HCC	HLA-DQA1/DRB1	rs9272105	1.28	1.22-1.35			[11]
		GRIK1*	rs455804	0.84	0.80-0.89			
Japanese, Korean	Chronic infection	HLA-DPA1	rs3077	0.46	0.39-0.54			[12]
		HLA-DPB1	rs9277542	0.50	0.43-0.60			
Chinese	Chronic infection	HLA-DPB1	rs9277535	0.60	0.51-0.70			[13]
		HLA-DPA1	rs3077	0.81	0.75-0.95			
		HLA-DQ	rs7453920	0.60	0.49-0.73			
	HCC	HLA-DQ	rs2856718	0.75	0.64-0.89			
		HLA-DQ	rs2856718	0.70	0.59-0.83			
		HLA-DPA1	rs3077	0.78	0.67-0.92			
Chinese	HCC	HLA-DQ	rs9275319	1.51	1.38-1.66			[14]
		STAT4*	rs7574865					

HLA: Human leukocyte antigen; SNP: Single nucleotide polymorphism; HCC: Hepatocellular carcinoma.

*DPA1* gene, including rs3077, and 7 SNPs related to *HLA-DPB1*, including rs9277535, were associated with chronic HBV infection<sup>[9]</sup>. Another study showed that rs7453920 and rs2856718 SNPs near *HLA-DQ* were associated with persistent HBV infection in addition to the rs3077 and rs9277535 SNPs<sup>[10]</sup> (Table 1).

A recent report from another Japanese group showed that rs3077 SNP near *HLA-DPA1* gene and rs9277542 SNP near *HLA-DPB1* gene were associated with persistent HBV infection<sup>[12]</sup>. Studies using genotyping methods validated that the rs3077 and rs2395309 SNPs near *HLA-DPA1* gene and the rs9277542 SNP near *HLA-DPB1* were associated with HBV infection in Han Chinese populations<sup>[23-25]</sup>.

GWAS revealed three independent variants within the *HLA* complex that were related to a poor response

to HB vaccine in the Indonesian population. Specifically, rs3135363 SNP near *HLA-DR*, rs9277542 SNP near *HLA-DPB1*, and rs9267665 in *HLA* class III were associated with antibody titers after HB vaccination<sup>[10]</sup>.

A comparison between cohorts with and without hepatocellular carcinoma (HCC) showed that rs9272105 SNP near *HLA-DQA1/DRB1* and rs455804 SNP near *GRIK1* were significantly associated with HCC development in Chinese patients with HBV<sup>[11]</sup>. There was a partial association of the genotype of rs9272105 to *HLA-DRB1\*0405* and *\*0901*. Another study showed that rs2856718 SNP at *HLA-DQ* and rs3077 SNP at *HLA-DPA1* had a protective effect against HCC progression as compared with the dominant SNP of rs2856718 in Han Chinese populations<sup>[13]</sup>. In 2013, it was reported that rs9275319 at *HLA-DQ* and rs7574865 at *STAT4* were

**Table 2** Single nucleotide polymorphisms within human leukocyte antigen loci associated with outcomes of hepatitis C virus infection

Ethnic group	Outcome	No. of cohorts	HLA locus	SNP	Odds	95%CI	Haplotype	Odds	Ref.
Japanese	HCC	721 HCC <i>vs</i> 2890 HCV-negative controls	MICA	rs2596542	1.34	1.16-1.53			[30]
Japanese	Cirrhosis	682 cirrhosis <i>vs</i> 1045 Chronic hepatitis	C6orf10	rs910049	1.73	1.40-2.15			[31]
			No gene	rs3135363	1.58	1.32-1.90			
							DQA1*0601	2.80	
							DPB1*0405	1.45	

SNP: Single nucleotide polymorphism; HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen; HCV: Hepatitis C virus.

independently associated with the risk of HCC in Han Chinese populations<sup>[14]</sup>. There was a moderate association between the genotype of rs9275319 SNPs with *HLA-DQB1\*0401* and *HLA-DQA1\*0303*. On the other hand, there was no significant association between HCC development by HBV infection and *HLA* alleles in Korean or Japanese populations<sup>[26]</sup>. It thus remains unclear whether specific HLA loci play important roles in hepatocarcinogenesis in patients with HBV.

### HCV infection

It is globally recognized that interleukin-28B (IL-28B) gene polymorphisms originally detected by GWAS are associated with spontaneous clearance of HCV, as well as with the response to combination therapy with pegylated interferon and ribavirin in patients with HCV<sup>[27,28]</sup>. However, this SNP is not located in HLA loci. A recent study identified rs4273729 SNP near *HLA DQB1\*0301* as a candidate allele for spontaneous clearance of HCV in populations with European and African ancestry<sup>[29]</sup>. *HLA DQB1\*0301* and *IL28B* are independently associated with spontaneous resolution of HCV infection.

Comparisons between cohorts with and without HCC showed that rs2596542 SNP at the 5' flanking region of *MICA* in *HLA* class III was significantly associated with HCC development in Japanese patients with HCV<sup>[30]</sup>. Soluble MICA levels in serum were significantly lower in AA genotype of rs2596542 and were associated with a high risk of HCC progression. The same group identified 2 SNPs in the *MHC* region that were associated with progression from chronic hepatitis to cirrhosis. These SNPs were located at rs910049 and rs3135363 on chromosome 6p21.3<sup>[31]</sup>. Imputation-based association analysis showed that *HLA-DQA1\*0601* and *HLA-DPB1\*0405* were associated with progression of cirrhosis (Table 2).

### FUTURE DIRECTIONS

Ongoing association studies are evaluating the effects of genetic variations on the outcomes of hepatitis virus infection in large groups of patients. However, most SNPs identified by association studies did not link to phenotype, and many other SNPs remained untyped. Imputation-based association analysis exploits information on patterns of multi-marker correlation ("linkage disequilibrium") from publically available databases to estimate ("impute") patient genotypes associated with

identified SNPs and thereby assess the relations of such genotypes to phenotypes<sup>[32,33]</sup>. Owing to this method, the relations between SNPs and *HLA* haplotypes associated with the outcomes of HBV or HCV infection are becoming clearer.

In HBV infection, conventional genotyping showed that *HLA* class II, DR and DQ haplotypes were the most important regions of host genetic factors for outcomes. However, GWAS showed that rs3077 SNP near *HLA-DPA1* gene and rs9277535 SNP near *HLA-DPB1* gene were associated with persistent HBV infection in Asian populations<sup>[7,9,12,13]</sup>. *HLA-DPA1* and *DPB1* have also been associated with responsiveness to HB vaccination<sup>[10,34,35]</sup>. To date, however, few studies have focused on *HLA-DP* because polymorphisms of *HLA-DP* haplotype do not vary greatly as compared with other loci of *HLA*<sup>[36]</sup>. The structures of *HLA-DP* and *HLA-DP* molecules are similar to those of other *HLA* class II molecules. Therefore, similar to the functions of other *HLA* class II molecules, *HLA-DP* and *HLA-DP* molecules might affect the ability of *HLA* class II molecules to present antigens to CD4-positive helper T cells and result in immune response to HBV. Recently, *HLA-DPA1* and *HLA-DPB1* mRNA expressions in normal liver were respectively associated with SNP types rs3077 and rs9277535 in European populations. The mRNA expressions of *HLA-DPA1* and *HLA-DPB1* were low in genotypes rs3077-G and 9277535-G, which were associated with a high risk of persistent HBV infection<sup>[37]</sup>. However, another study in European- and African-Americans showed that rs9277534 of the *HLA-DPB1* allele (496-A/G) was a novel variant associated with persistent HBV infection<sup>[38]</sup>. In contrast to the former study, the 496-GG genotype was associated with both higher mRNA expression of *HLA-DP* and persistent HBV infection.

Inconsistent results have been obtained for the association between *HLA* alleles and HCC in patients with HBV infection. In Han Chinese populations, several SNPs in *HLA* class II have been associated with progression of HCC. However, no common SNP was confirmed by independent researchers. In addition, SNPs in chromosomes 1p36.22, 2q32.2, and 21q21.3, were also associated with HBV-related HCC<sup>[39]</sup>. Further examinations are definitely required to elucidate the role of *HLA* loci on the progression of HCC in patients with HBV.

GWAS indicated that *HLA* loci are related to important host factors involved in several aspects of HCV

infection. First, *HLA DQB1\*0301* was reported to be independently associated with spontaneous clearance of HCV infection. Previous HLA haplotype analysis showed that *HLA DQB1\*0301* was associated with HCV clearance in French females, African-Americans, and Italian populations<sup>[40-42]</sup>. Thus, GWAS confirmed the results of previous results. However, the mechanism by which such alleles affect HCV clearance remains undetermined.

In the Japanese population, rs4273729 SNP near *HLA DQB1\*0301* and *MICA* SNP in - *HLA* class III were respectively associated with progression of hepatitis to cirrhosis<sup>[31]</sup> and HCC<sup>[30]</sup> in patients with HCV. This is attractive information for the prediction of clinical course, but several issues remain to be defined. First, HCC most frequently develops in cirrhotic patients infected with HCV. It is not known why different SNPs are identified in continuous pathological conditions such as HCC and hepatic cirrhosis in patients with HCV. Next, an intronic SNP in the *DEPDC-5* gene, without an *HLA* locus, was also associated with HCC development in the Japanese population<sup>[43]</sup>. In European populations, several SNPs without *HLA* loci were associated with the progression of hepatic fibrosis<sup>[44]</sup>. The progression of chronic hepatitis C has been confirmed to depend on multiple factors, including age, gender, infection period, obesity, alcohol intake, and treatment<sup>[45]</sup>. It is suspected that the effects of *HLA* loci on fibrosis progression or the development of HCC (or both) differ in the each population studied.

In conclusion, genome association analysis of large numbers of cohorts indicated that *HLA* loci are one of the most important host determinants of the clinical characteristics of HBV and HCV infections, acting in conjunction with factors such as viral load, viral genotype, age, alcohol intake, and hepatic fibrosis. However, it is necessary to validate reported SNPs on *HLA* loci in global populations and to elucidate *HLA*-allele-regulated molecular responses to hepatitis virus infection.

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## What does irritable bowel syndrome share with non-alcoholic fatty liver disease?

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### Abstract

Non-alcoholic fatty liver disease (NAFLD) and irritable bowel syndrome (IBS) are two very common diseases in the general population. To date, there are no studies that highlight a direct link between NAFLD and IBS, but some recent reports have found an interesting correlation between obesity and IBS. A systematic PubMed database search was conducted highlighting that common mechanisms are involved in many of the local and systemic manifestations of NAFLD, leading to an increased cardiovascular risk, and IBS, leading to microbial dysbiosis, impaired intestinal barrier and altered intestinal motility. It is not known when considering local and systemic inflammation/immune system activation, which one has greater importance in NAFLD and IBS pathogenesis. Also, the nervous system is implicated. In fact, inflammation participates in the development of mood disorders, such as anxiety and depression, characteristics of obesity and consequently of NAFLD and, on the other hand, in intestinal hypersensitivity and dysmotility.

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**Key words:** Non-alcoholic fatty liver disease; Irritable bowel syndrome; Low grade chronic inflammation; Cytokines

**Core tip:** The link between non-alcoholic fatty liver disease (NAFLD) and irritable bowel syndrome (IBS) should be carefully evaluated in future research, representing an intriguing field of investigation. A better understanding of the role of systemic inflammation and activation of the immune system may be necessary to clarify obscure points of NAFLD and IBS pathogenesis, and therefore it can be helpful in the development of new therapies.

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### INTRODUCTION

Fat accumulation in the liver in the absence of specific causes of hepatic steatosis, such as alcohol consumption, with or without liver inflammation and its consequences, is described as non-alcoholic fatty liver disease (NAFLD)<sup>[1]</sup>. To date, there are no studies that highlight the link between NAFLD and irritable bowel syndrome (IBS), but some recent reports have found an interesting correlation between obesity and IBS. A relationship between body mass index (BMI) and IBS-like symptoms seems to exist<sup>[2,3]</sup>. Moreover, in IBS subjects a high BMI is associated with significantly faster colonic and recto-sigmoid transit and high stool frequency<sup>[4]</sup>. Cremonini *et al*<sup>[5]</sup> have compared obese binge eaters and non binge eaters to healthy controls and have evidenced that obese subjects more frequently have constipation, diarrhea, straining and flatus regardless of the eating disorder, and that obese binge eaters are characterized by more recurrent upper

and lower gastrointestinal symptoms. Clements *et al*<sup>[6]</sup> have highlighted that obese patients experience more severe gastrointestinal symptoms than healthy controls, and that after laparoscopic Roux-en-Y gastric bypass they have increased abdominal pain, gastroesophageal reflux disease, sleep disturbance and IBS symptoms. The development of small intestinal bacterial overgrowth (SIBO) may explicate, perhaps partially, the incidence of IBS symptoms in obese subjects with previous abdominal surgery, and in this case the bacterial overgrowth may be the consequence of changes in the function and in the morphological structure of the gut<sup>[7]</sup>.

NAFLD pathogenesis is strictly allied to metabolic syndrome, insulin resistance and obesity<sup>[8,9]</sup> but inflammation plays an equally important role. Day *et al*<sup>[10]</sup> have developed in 1998 the classical “two-hit” theory: liver fat accumulation is the “first-hit”, linked to obesity, insulin resistance and metabolic syndrome, while the “second-hit” is activated by hepatic inflammation, together with oxidative stress and endotoxemia, which have a key role in the progression to non-alcoholic steatohepatitis (NASH) and, over time, to fibrosis, until the development of cirrhosis<sup>[11]</sup>.

Actually, this classic view has been revised, because a frank distinction between “first-hit” and “second-hit” is not easy to make, leading to the “multiple-hit theory”<sup>[12]</sup>. Recent studies have shown that, independently from fat accumulation in the liver, obesity systemically leads to activation of the immune system and low-chronic inflammation from the first stage of the disease<sup>[13]</sup>.

Obesity and hepatic fat accumulation are hypothetically implicated in IBS genesis or development. Moreover, an initial correlation between IBS and NAFLD can be suggested by some interesting data. In the pathogenesis of NAFLD and NASH, there is a strong involvement of the gastrointestinal system, as evidenced by many studies on the so-called “gut-liver axis”, aiming to comprehend the role of gut microbiota, SIBO and intestinal permeability dysfunction<sup>[14]</sup>. On the other hand, hepatic fat accumulation and hepatic inflammation in NAFLD subjects<sup>[15]</sup> and gastrointestinal symptoms in IBS subjects<sup>[16]</sup> both improve after therapy with probiotics.

IBS, one of the most common gastrointestinal disorders with an estimated prevalence of 7%-10% worldwide<sup>[17]</sup>, is characterized by abdominal pain/discomfort, changes in bowel habits and no association with organic cause. Despite the fact that in IBS subjects macroscopically evident pathological lesions at colonoscopy have not been found, molecular biology and in-depth histological investigations have revealed the activation of the immune system. A key piece of evidence is that the exposure of rodent<sup>[18-20]</sup> or human<sup>[21]</sup> tissues or cell cultures<sup>[22]</sup> to mucosal or luminal mediators from IBS subjects leads to impaired nervous stimulation or intestinal barrier damage. A proportion of patients develop IBS symptoms after infectious gastroenteritis, or in a remission state from microscopic colitis, Crohn's disease and ulcerative colitis, or on a gluten-free diet for celiac disease<sup>[23]</sup>.

Inflammation and immune system activation may be the mechanisms linking two apparently very different diseases, and the purpose of our review is to collect key evidence supporting their relationship and therefore to explain the pathophysiological link between the intestine and the liver, which is exquisitely firstly anatomical and consequently also functional.

## IMMUNITY IN NAFLD

A low-grade chronic inflammation underlies all NAFLD entities/stages and can develop and promote the liver damage<sup>[13]</sup>.

Innate and adaptive immune pathways are activated in obesity and many findings show that adipose tissue inflammation exacerbates hepatic steatosis and promotes non-alcoholic steatohepatitis (NASH). Obese individuals more frequently develop infectious diseases<sup>[24-26]</sup> as complications after surgery<sup>[27,28]</sup>, and an increased BMI is associated with enhanced risk of infections in institutionalized geriatric patients<sup>[29]</sup>.

The adipose tissue has an important role in regulating energy utilization, vascular functions and immune system homeostasis<sup>[30]</sup>. C-reactive protein (CRP)<sup>[8]</sup>, interleukin (IL)-6<sup>[31]</sup>, fibrinogen and plasminogen activator inhibitor-1<sup>[32]</sup> levels are higher in obese patients compared to healthy subjects. Stanton *et al*<sup>[33]</sup> have recently found that obese mice, after high fat and high cholesterol diets, express abnormal levels of macrophages and inflammation-associated genes in adipose tissue and in liver.

Obesity can influence liver metabolism directly, *via* circulating free fatty acids (FFA), and indirectly, *via* pro-inflammatory cytokine production. FFA and other lipids in hepatocytes are involved in production of reactive oxygen species, mitochondrial dysfunction and endoplasmic reticulum stress. They have proapoptotic capacity and can stimulate proinflammatory signaling pathways<sup>[30]</sup>. FFA from adipose tissue, food and intestinal bacteria can bind toll like receptors (TLR) expressed on immune cells systemically and also in the liver, and enhance the hepatic expression of TLR-4 and TLR-2<sup>[34]</sup>, these being receptors fundamental to the activity of immune system.

The presence of a dysregulation of the immune system in NAFLD has been firstly evidenced by the modification in immune cell populations in the liver. Natural killer (NK) cell circulating levels are reduced in obese rats<sup>[35]</sup>; meanwhile in the liver of NASH subjects their concentration is increased<sup>[36]</sup>. These cells have anti-fibrotic effects and produce apoptosis directly<sup>[37]</sup> and *via* interferon gamma (IFN $\gamma$ ) production<sup>[38]</sup> from hepatic stellate cells (HSC), which have a major role in liver fibrosis<sup>[39]</sup>. In the light of the strict resemblance between NASH and alcoholic hepatitis, Jeong *et al*<sup>[40]</sup> have detected that alcohol contributes to the anti-fibrotic effect of IFN $\gamma$  and NK cells in animals.

Another immune cell population, natural killer T (NKT) cells, which express NK cell markers and  $\alpha/\beta$  T cell receptors, are reduced in steatotic, obese mice<sup>[41,42]</sup>.

and in humans<sup>[43]</sup>. NKT cells are able to produce both T helper (Th) 1 and Th2 cytokines but their depletion in NAFLD has been associated with Th1 polarization of hepatic T cells in mice<sup>[44,45]</sup>.

Two T helper cell subsets were recently discovered and are strictly related to the innate immune response. Th17 cells on one side and Treg cells on the other balance tolerance and elicitation of immune responses<sup>[46]</sup>. Th17 cells produce IL-17, IL-21 and IL-22, and require transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-6 for their differentiation<sup>[47]</sup>, the same cytokines that inhibit Treg cells. A Chinese study group has recently discovered that oxidative stress induces Treg cell apoptosis in mice with fatty livers<sup>[48]</sup> and subsequently has found also that Th17 cells are increased in the liver of animal and human NASH models<sup>[49]</sup>.

Kupffer cells (KC) are liver macrophages involved in the response to such stressors as infections, ischemia and toxins<sup>[50]</sup> and they are also implicated in liver inflammation and NASH progression<sup>[51]</sup>.

Tumor necrosis factor (TNF) - $\alpha$ , a cytokine produced by KCs, hepatocytes, and abdominal fat, is associated with the development in rodents<sup>[52,53]</sup> and in humans<sup>[54-57]</sup> of insulin resistance, NAFLD and NASH. The role of TNF- $\alpha$  in NAFLD may be due to its capacity to induce hepatocyte apoptosis, insulin resistance and to regulate KC activation locally<sup>[58,59]</sup>. Moreover, TNF- $\alpha$  regulates hepatic lipid metabolism<sup>[60]</sup>.

In a NASH animal model involving choline-deficient diet fed rats it was found that there was an increase in serum and portal alanine aminotransferase levels and hepatic TNF- $\alpha$ , IFN $\gamma$  and TLR4. Higher TNF- $\alpha$  levels were detected in KCs and, most importantly, increased TNF- $\alpha$ , TLR4 expression, and macrophage/dendritic cell populations were found in ileal tissue specimens, demonstrating also the involvement of the gut in steatotic liver damage<sup>[61]</sup>.

To date, it is debatable whether circulating levels of TNF- $\alpha$  may discriminate the presence of NAFLD in obese subjects or in subjects with metabolic syndrome<sup>[62,63]</sup>, but they seem to be useful in the non-invasive diagnosis of hepatic fibrosis in NASH<sup>[64]</sup>.

IL-6 is a polyvalent cytokine with proinflammatory and prooncogenic activity, and it supports hematopoiesis<sup>[65]</sup> and is a predictive marker of insulin resistance and cardiovascular diseases<sup>[66]</sup>. In animal<sup>[67]</sup> and human<sup>[68,69]</sup> models respectively, hepatic and serum IL-6 levels are higher in NAFLD. Initially this cytokine was considered hepatoprotective because it reduces oxidative stress and prevents mitochondrial dysfunction in animal models<sup>[70,71]</sup>. Moreover, there are contrasting data on IL-6 production in the liver of NAFLD subjects<sup>[57,72]</sup>. IL-6, with TNF- $\alpha$ , suppresses adiponectin levels; meanwhile, TNF- $\alpha$  stimulates the production of leptin<sup>[73,74]</sup>. Adiponectin is an adipocytokine with anti-inflammatory properties and it decreases in subjects with increased liver fat concentration<sup>[75]</sup>. Leptin has opposite effects; it activates neutrophils and innate immune system<sup>[76]</sup>, is associated with obesity and may contribute to NAFLD progression<sup>[77]</sup>. IL-6 production is also enhanced by TNF- $\alpha$  and IL-1 and can act

with paracrine and endocrine mechanisms to activate IL-6 signaling systemically and peripherally in other organs such as liver and muscle<sup>[13]</sup>. FFA and IL-17 synergistically induce IL-6 production; on the other hand IL-6, with TGF- $\beta$ 1, enhances Th17 response in *in vitro* HepG2 cell models<sup>[49]</sup>. Tarantino *et al*<sup>[78]</sup> have also observed that, surprisingly, NAFLD subjects have increased TGF- $\beta$ 1 blood levels compared with those with chronic hepatitis C.

An anti-inflammatory cytokine, IL-10, is protective for hepatic steatosis, as seen in IL-10 deficient mice<sup>[79]</sup> as well as in NAFLD humans<sup>[80]</sup>, and the inhibition of IL-10 promotes hepatic steatosis, enhances the expression of proinflammatory cytokines and impairs insulin signal transduction<sup>[81]</sup>. Main data on the pathophysiological role of inflammatory cytokines in NAFLD are summarized in Table 1.

Brun *et al*<sup>[82]</sup> have observed that HSCs isolated from genetically obese and diabetic mice show more pronounced fibrogenic responses induced by lipopolysaccharide (LPS) than HSCs from lean animals. Thus, HSCs are more sensitive to bacterial endotoxins, because genetically obese mice have an impaired intestinal permeability leading to increased portal endotoxemia. To expand on the evidence that systemic inflammation is also related to intestinal inflammation, a recent study undertaken by Kant *et al*<sup>[83]</sup> has found that weight loss in obese subjects reduces fecal calprotectin levels. Precedent studies have pointed out that circulating calprotectin levels are related to increased BMI<sup>[84,85]</sup>. As detailed later, the intestine, and especially intestinal inflammation, is closely related to NAFLD pathogenesis.

## IMMUNITY IN IBS

In IBS subjects a low chronic inflammation is present and many other immune phenomena are also points of contact with hepatic steatosis.

The intestinal mucosa physiologically contains immune cells much more than other organs and tissues, and this is mainly due to its anatomical configuration and function as the first barrier of the organism<sup>[86]</sup>. In the "irritated" gut there is an increased population of immune cells in the small and large intestine, as reported in many studies<sup>[87,88]</sup>. Moreover, the inflammatory infiltrate is lower than in ulcerative colitis (UC) but is similar to that revealed in microscopic colitis<sup>[89]</sup>. These findings, with others discussed later, lead to the theory that IBS could be considered as an inflammatory disease.

The adaptive immune system is involved in the low grade inflammation of the gut, specifically, CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cell count is increased<sup>[89-91]</sup> in the gut and in the peripheral blood of IBS subjects.

The innate immune response is also implicated in IBS pathogenesis. An increased number of mast cells are found in the small<sup>[92]</sup> and large<sup>[93]</sup> intestine. These cells are in close contact with enteric nerve endings<sup>[94]</sup> and this is an important factor in the neuronal stimulation that underlies the establishment of typical IBS symptoms<sup>[95]</sup>. Braak *et al*<sup>[96]</sup> are discordant on this point because they

**Table 1** Principal findings on inflammatory cytokines in non-alcoholic fatty liver disease in humans, and in *in vitro* and animal models

Principal findings
<p><b>TNF-<math>\alpha</math></b></p> <p><i>In vitro</i>: FFA induce <i>TNF-<math>\alpha</math></i> gene expression<sup>[60]</sup>. KC and hepatocytes from NAFLD produce <math>\uparrow</math> <i>TNF-<math>\alpha</math></i> and <math>\uparrow</math> lipid peroxidation and accumulation<sup>[59,61]</sup>. <i>TNF-<math>\alpha</math></i> induces hepatocyte apoptosis<sup>[59]</sup></p> <p>Animal: <i>TNF-<math>\alpha</math></i> regulates KC apoptosis<sup>[58]</sup>. Hepatic, portal blood and intestinal <i>TNF-<math>\alpha</math></i> is <math>\uparrow</math><sup>[52,53,61]</sup></p> <p>Human: Circulating levels are <math>\uparrow</math> in NAFLD and NASH<sup>[57,68]</sup>. Contrasting data on simple FL<sup>[55,62]</sup>. They correlate with activity and progression of NAFLD<sup>[64]</sup> But do not differentiate mild to severe NASH<sup>[60]</sup>. NASH subjects have also <math>\uparrow</math> PBMCs <i>TNF-<math>\alpha</math></i>, IL-6 and IL-8 production<sup>[68]</sup>. <i>TNF-<math>\alpha</math></i> mRNA expression is <math>\uparrow</math> in liver and fat of NASH compared with NAFLD<sup>[57]</sup>, but there are contrasting data<sup>[55,238]</sup> <i>TNF-<math>\alpha</math></i> polymorphism is most frequent in NAFLD and correlates also with IR<sup>[56]</sup></p>
<p><b>IL-6</b></p> <p><i>In vitro</i>: FFA induces IL-6 expression in hepatic cell cultures<sup>[72]</sup> and enhances Th17 response<sup>[49]</sup></p> <p>Animal: IL-6, <i>TNF-<math>\alpha</math></i>, IL-8 production is <math>\uparrow</math> in liver and muscle of NAFLD mice<sup>[64]</sup>. Possible hepatoprotective role<sup>[70,71]</sup></p> <p>Human: <math>\uparrow</math> IL-6 blood levels and other inflammatory and cytonecrosis indexes in NAFLD and NASH subjects compared to controls and obese<sup>[57,68,69]</sup>. IL-6 is an index of NASH activity and progression<sup>[72]</sup>. Normal levels of IL-6 and normal spleen longitudinal diameter may be useful in excluding NASH from NAFLD<sup>[34]</sup>. IL-6 tissue expression is controversial in liver of NAFLD<sup>[57,72]</sup></p>
<p><b>IL-8</b></p> <p><i>In vitro</i>: IL-8 with <i>TNF-<math>\alpha</math></i> are <math>\uparrow</math> in NAFLD and in NASH compared to FL<sup>[64]</sup>. FFA induces IL-8 expression<sup>[60]</sup></p> <p>Human: Blood levels of IL-8, IL-6 and <i>TNF-<math>\alpha</math></i> are <math>\uparrow</math> in NASH<sup>[68,69]</sup></p>
<p><b>IL-1<math>\beta</math></b></p> <p>Animal: NAFLD rats express similar IL-1<math>\beta</math>, <i>TNF-<math>\alpha</math></i> and IL-6 levels in liver and in muscle<sup>[64]</sup></p> <p>Human: <i>TNF-<math>\alpha</math></i>, IL-6 and IL-1<math>\beta</math> blood levels are <math>\uparrow</math> in NAFLD and NASH<sup>[68,69]</sup></p>
<p><b>TGF-<math>\beta</math>1</b></p> <p><i>In vitro</i>: IL-17 and FFA induce IL-6 in hepatocytes and IL-6, with TGF-<math>\beta</math>1, enhance Th17 response<sup>[49]</sup></p> <p>Human: TGF-<math>\beta</math>1 blood levels in NAFLD are <math>\uparrow</math> than CHC<sup>[78]</sup></p>
<p><b>IL-10</b></p> <p>Animal: After IL-10 inhibition, <i>TNF-<math>\alpha</math></i>, IL-6 and IL-1<math>\beta</math> levels increase in liver of HFD mice<sup>[81]</sup>. IL-10 knock-out mice have <math>\uparrow</math> FFA plasma levels and hepatic TG<sup>[79]</sup></p> <p>Human: In NAFLD and obese children, lower IL-10 blood levels correlate with markers of visceral and subcutaneous fat, insulin, HOMA-IR, ALT, AST and GGT<sup>[77]</sup></p>
<p><b>IL-17</b></p> <p><i>In vitro</i>: IL-17 and FFA induce IL-6 production<sup>[49]</sup></p> <p>Animal: LPS-induced liver injury ameliorated after IL-17 blockade in HFD rats<sup>[49]</sup></p> <p>Th2 cytokines (IL-4, IL-5, IL-13)</p> <p>Animal: Rats genetically oriented to a Th1 response develop steatosis and lobular inflammation more than others oriented to Th2 response<sup>[44,45]</sup></p>

TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; FFA: Free fatty acids; KC: Kupffer cells; NAFLD: Non-alcoholic fatty liver disease; NASH: Non alcoholic steatohepatitis; FL: Fatty liver; PBMCs: Peripheral blood mononuclear cells; IL: Interleukin; IR: Insulin resistance; TGF-1 $\beta$ : Tumor growth factor 1  $\beta$ ; Th17: T helper 17; CHC: Chronic hepatitis C; HFD: High fat diet; TG: Triglycerides; HOMA-IR: Homeostasis model of assessment-insulin resistance; ALT: Alanine-aminotransferase; AST: Aspartate-aminotransferase; GGT:  $\gamma$ -Glutamyltransferase; LPS: Lipopolysaccharide; Th2: T helper 2; Th1: T helper 1.

have observed a decreased number of mast cells, macrophages and T cells in IBS subjects. Moreover, they do not find visceral hypersensitivity or abnormal stress response.

Few reports have examined other immune cells involved in the innate immune system in IBS. NK cells<sup>[97]</sup> and neutrophils<sup>[98]</sup> may be hyper-activated but, to determine their role in intestinal inflammation, more studies are needed.

Contrasting data are reported on the monocyte/macrophage population. These cells were reduced<sup>[99]</sup> or normal<sup>[90]</sup> in number in the gut of IBS patients compared to controls but they may be hyper-activated, as seen by increased calprotectin expression<sup>[90]</sup>. Calprotectin is a calcium-binding protein produced by phagocytes with pro-inflammatory activity, such as leukocyte recruitment<sup>[100]</sup>. Fecal calprotectin may be useful in the differential diagnosis between inflammatory bowel diseases (IBD) and IBS<sup>[101]</sup>. Moreover, other authors have observed that patients with IBD and IBS-like symptoms have significantly higher fecal calprotectin levels than those with IBD but without IBS symptoms<sup>[102]</sup>. Shulman *et al.*<sup>[103]</sup> have shown that fecal calprotectin concentration is greater in children with IBS and functional abdominal pain compared to

controls, and also in the same population there is an impaired permeability in the proximal and distal gut.

There are contrasting data on the role of Treg cells, a T cell subpopulation with regulatory functions in IBS: these cells seem to be normally or under-expressed in intestinal tissues and blood of IBS subjects<sup>[104,105]</sup>, even though previously Chadwick *et al.*<sup>[88]</sup> have observed increased CD25<sup>+</sup> T cell population in the lamina propria of IBS subjects. The role of Th17 cells in the pathophysiology of IBS is still unexplored but, recently, Andoh *et al.*<sup>[106]</sup> have summarized the main evidence on the role of this subpopulation in intestinal inflammation. It would be interesting to see if IBS might be involved in the dysregulation between Th17 and Treg cells as shown in NAFLD.

Studies on proinflammatory cytokine production in IBS have evidenced the activation of both the innate and adaptive immune systems. Indeed, different study methods were used to explore the systemic cytokine production and results were not always concordant<sup>[107]</sup>.

IL-6 and TNF $\alpha$  are the most studied inflammatory cytokines in IBS. In many reports blood levels of TNF $\alpha$  and IL-6 are increased<sup>[108-112]</sup>. Similar results are reported in cultured peripheral blood mononuclear

**Table 2 Principal findings on inflammatory cytokines in irritable bowel syndrome in humans, and in *in vitro* and animal models**

Principal findings
<p><b>TNF-<math>\alpha</math></b></p> <p>Animal: D-IBS supernatants have <math>\uparrow</math> levels of proinflammatory cytokines and they cause hypersensitivity in mouse colonic afferent endings<sup>[122]</sup></p> <p>Human: IBS has <math>\uparrow</math> circulating TNF-<math>\alpha</math> levels<sup>[109,112]</sup>, especially D-IBS<sup>[112]</sup> or in patients with comorbidities such as fibromyalgia, premenstrual dysmorphic disorder and chronic fatigue syndrome<sup>[109]</sup>. Baseline and LPS-stimulated levels in PBMCs of proinflammatory cytokines as TNF-<math>\alpha</math>, in IBD and D-IBS, are <math>\uparrow</math> and are related to symptom intensity<sup>[108]</sup>. TLR-2, TLR-4 and TLR-5 antagonists induce TNF-<math>\alpha</math> production<sup>[128]</sup>. No difference in TNF-<math>\alpha</math> and other proinflammatory cytokine production (IL-6 and IL-1<math>\beta</math>) in the gut of IBS subjects compared to controls<sup>[116]</sup></p>
<p><b>IL-6</b></p> <p><i>In vitro</i>: No differences in colonic production between IBS and controls 116. IL-6 have excitatory action on colonic cells from IBS rats producing neuronal activation and absorption/secretory responses<sup>[115]</sup></p> <p>Animal: IL-6 colonic secretion is <math>\uparrow</math> in IBS rats and activate submucosal neurons<sup>[127]</sup></p> <p>Human: IL-6 blood levels are <math>\uparrow</math> in all IBS subtypes<sup>[109-111]</sup>. IL-6 levels are related to ACTH response and <math>\Delta</math>ACTH/<math>\Delta</math>Cortisol ratio<sup>[110]</sup>. Baseline and LPS or TLR agonist-stimulated PBMC levels are <math>\uparrow</math> in IBS<sup>[108]</sup></p>
<p><b>IL-8</b></p> <p><i>In vitro</i>: Reduced expression of mRNA of IL-8 in <i>ex vivo</i> biopsy cultures<sup>[116]</sup></p> <p>Human: Circulating levels of IL-8 are <math>\uparrow</math> in IBS<sup>[109-111,119]</sup>. TLR-3 and TLR-7 agonists induce IL-8 production in PBMCs<sup>[128]</sup></p>
<p><b>IL-1<math>\beta</math></b></p> <p>Animal: In stressed rats with previous acute colitis IL-1<math>\beta</math> mRNA expression is <math>\downarrow</math><sup>[117]</sup></p> <p>Human: <math>\uparrow</math> IL-1<math>\beta</math> levels in IBS<sup>[108,128]</sup>, in C-IBS and in D-IBS<sup>[108]</sup>. With TNF-<math>\alpha</math>, IL-1<math>\beta</math> <math>\uparrow</math> levels are found in IBS subjects with fibromyalgia, premenstrual dysmorphic disorder and chronic fatigue syndrome<sup>[109]</sup>. IL-1<math>\beta</math> <math>\uparrow</math> production in PBMCs stimulated by antiCD3/CD28 antibody<sup>[91]</sup> and by TLR-4 and TLR-5 agonists<sup>[128]</sup>. Increased IL-1<math>\beta</math> expression in rectum of PI-IBS<sup>[121]</sup></p>
<p><b>TGF-1<math>\beta</math></b></p> <p>Animal: No different expression of TGF-<math>\beta</math>1 protein in colon of IBS rats<sup>[11]</sup></p> <p>Human: TGF-1<math>\beta</math> intermediate producers may be at risk of developing IBS<sup>[114]</sup></p>
<p><b>IL-10</b></p> <p>Human: IBS subjects have <math>\downarrow</math> circulating levels of IL-10<sup>[112]</sup>. Altered IL-10/IL-12 ratio in PBMCs with Th1 proinflammatory state<sup>[113]</sup>. IL-10 levels are <math>\downarrow</math> and IFN<math>\gamma</math> levels are <math>\uparrow</math> in colon of PI-IBS compared to non PI-IBS and controls<sup>[119]</sup>. IL-10 high producer genotype is protective against IBS<sup>[114]</sup></p>
<p><b>Th2 cytokines (IL-4, IL-5, IL-13)</b></p> <p>Animal: Th2 cytokines may have a role in intestinal hypercontractility<sup>[123]</sup></p> <p>Human: Stimulated PBMCs IL-5 and IL-13 levels are <math>\uparrow</math> in IBS<sup>[124]</sup></p>

TNF- $\alpha$ : Tumor necrosis factor  $\alpha$ ; D-IBS: Diarrhoea-predominant irritable bowel disease (IBS); IBD: Inflammatory bowel disease; LPS: Lipopolysaccharide; PBMCs: Peripheral blood mononuclear cells; TLR: Toll like receptor; IL: Interleukin; ACTH: Adrenocorticotrophic hormone; C-IBS: Constipation-predominant IBS; PI-IBS: Post-infectious IBS; TGF-1 $\beta$ : Tumor growth factor 1  $\beta$ ; IFN $\gamma$ : Interferon  $\gamma$ ; Th2: T-cell mediated helper response.

cells<sup>[108,111]</sup>. Studies on Peripheral blood mononuclear cells (PBMCs) have also noticed decreased levels of the anti-inflammatory IL-10<sup>[112,113]</sup>, in agreement with the systemic inflammatory state in IBS. Moreover, the IL-10 high producer genotype seems to be protective against IBS, whereas IL-10 low producer, and maybe even TGF-1 $\beta$  intermediate producer genotypes, are a risk factor for IBS development<sup>[114]</sup>. In IBS mice, IL-6 may enhance colonic cells neuronal activation and their absorption/secretory responses<sup>[115]</sup>. The intestinal cytokine production is poorly understood<sup>[116-119]</sup>, and, as described in a recent review by Ortiz-Lucas *et al.*<sup>[120]</sup>, only IL-1 $\beta$  expression is clearly increased in post-infectious IBS (PI-IBS)<sup>[121]</sup>. On the contrary, Hughes *et al.*<sup>[122]</sup> have observed increased cytokine expression in supernatants of mice with IBS and that visceral neurons express receptors for IL-6, TNF- $\beta$ , IL-1 $\beta$  and IL-10, confirming the role of these pro-inflammatory cytokines in gut homeostasis.

Th 2 cytokines were also considered in recent reports: in animals Th 2 cytokines enhance intestinal motility<sup>[123]</sup> and in IBS subjects stimulated PBMCs produce more IL-5 and IL-13 than controls<sup>[124]</sup>.

Cytokines have several roles in the development of IBS symptoms. For example, TNF- $\alpha$  can act on the peripheral nervous system as well as on the central nervous system (CNS) to develop a symptom burden of hyper-

sensitivity, nausea, emesis, gastric hypomotility, anorexia and fever<sup>[125,126]</sup>. IL-6 is able to stimulate submucosal neurons in IBS animal models<sup>[127]</sup>, most probably *via* a TLR-mediated mechanism<sup>[128]</sup>. TNF- $\alpha$  and IL-6 are also implicated in intestinal barrier integrity<sup>[129]</sup> (Table 2).

## NAFLD AND IBS MAY BE RELATED

The above-mentioned evidence suggests that innate immunity is a main pathogenetic component of both NAFLD and IBS. But, how does the immune system work in patients with both NAFLD and IBS? In other words, is the similar action of pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$ , the only one that can be found on the immune system side?

The metabolic syndrome, which often anticipates or is detected in conjunction with NAFLD, leads to a state of chronic inflammation, systemic or local (hepatic)<sup>[12]</sup>, but to date it is still unclear which one of the two types has a greater impact on these patients, even if a lot of evidence favors the former<sup>[13]</sup>. A very similar scenario, but with partly different participants, is possible in IBS. Although the disease has not been overtly related to an inflammatory systemic disease, as happens for the metabolic syndrome, nevertheless, IBS is characterized by hyper-activation of the immune system and general inflammation. Indeed, many

researchers have struggled to find a similar component at local level, studying the intestinal cytokine production, but they have not always had a favorable outcome<sup>[107,120]</sup>. In some subsets of IBS patients, such as diarrhoea-predominant IBS (D-IBS) and IBS developing following infective gastroenteritis (PI-IBS), there is often a frank intestinal inflammation<sup>[108,119]</sup>. On the contrary, in C-IBS a systemic inflammation is not always associated with a local counterpart or is less apparent than in D-IBS<sup>[122]</sup>.

NAFLD and IBS are classically defined as different diseases. NAFLD is related to the metabolic syndrome, obesity, diabetes and insulin resistance and IBS is a functional intestinal disease closest to psychological disorders such as depression and anxiety, certainly not to liver diseases. But, surprisingly, there are many points of contact, such as the dysfunction of the intestinal microbiota, the impaired intestinal barrier, intestinal dysmotility and brain-gut axis dysfunction, which are fundamental to their pathogenesis, being related to the immune activation and inflammation.

Thus, principal questions are: Can metabolic liver disease affect the functions of the gastrointestinal tract leading to syndromic manifestations typical of IBS? and may the bowel dysfunction lead or otherwise support the development of a chronic hepatic inflammatory state?

## GUT MICROBIOTA

The gut microbiota is a composite member of our body. Intestinal bacteria interact with the intestinal epithelial barrier and subsequently with extraintestinal organs performing physiological and pathological actions.

This close contact makes the microbiota important for the metabolism of nutrients and energy delivery<sup>[130]</sup>, the intestinal barrier function<sup>[131]</sup>, the natural tropism of the intestinal wall<sup>[132]</sup> and ensures the maturation of intestinal immune tolerance and the immune response<sup>[133]</sup>.

The dysregulation of the intestinal bacterial milieu is a component of NAFLD and IBS. Recent reports have also shown both in NAFLD and in IBS an important role for TLR. These are receptors that characterize the innate immunity and link specific molecules such as pathogen-associated molecular patterns, LPS, and danger-associated molecular patterns<sup>[134]</sup>. These receptors are able to elicit the innate immune response once activated (they induce the expression of proinflammatory chemokines, cytokines and adhesion molecules on immune cells)<sup>[135]</sup>. In NAFLD and in IBS this role is consistently related to the alteration of gut microbiota, impaired intestinal permeability and impaired intestinal motility<sup>[136,137]</sup>.

Changes in microbiota composition and simultaneous or subsequent dysregulation of intestinal permeability let PAMPs and TLRs be in strict contact in the deeper layers of the intestinal wall and thus lead to stimulation of the innate immune response<sup>[138]</sup>.

Despite the fact that the roles of TLRs in the liver of NAFLD and NASH are well established<sup>[137]</sup>, only recently have the activity of TLRs in IBS been studied. Ohman

*et al.*<sup>[139]</sup> have observed increased expression of TLR2 on circulating monocytes in IBS. A study from McKernan *et al.*<sup>[128]</sup> demonstrated that the TLR-induced cytokine release (IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ ) was enhanced in blood from IBS subjects. The TLR mRNA production in the gut mucosa of mice with colonic visceral hypersensitivity was studied and significant increases were seen<sup>[140]</sup>. Similar results were found in humans<sup>[141]</sup>.

TLRs are fundamental in T-cell differentiation and activation, particularly for Th17 and Treg cells<sup>[142]</sup>. In the gut, bacterial products<sup>[143]</sup>, acute phase proteins<sup>[144]</sup> and proinflammatory cytokines such as IL-6 and TGF- $\beta$ <sup>[145]</sup> promote Th17 response, meanwhile IL-25 and IL-23<sup>[146]</sup> produced by epithelial cells inhibit it.

## Obesity and NAFLD

In the literature there are few reports on the intestinal microbiota composition in NAFLD. The role of intestinal dysbiosis in these patients may be assumed by reports on microbiota present in obese subjects or by indirect data on the action of bacterial products from the gut delivered to the liver in NAFLD.

Obese patients are characterized by low intestinal bacterial diversity. They have a reduced *Bacteroides* and increased *Firmicutes* population compared to controls, and this proportion improves with weight loss<sup>[147]</sup>. Studying the microbiome, the same group has found that obese patients exhibit impaired bacterial gene expression<sup>[148]</sup>.

Animal models have shown that the intestinal microbiota may have an important role in energy harvesting and fat storage. Germ-free mice seem to be protected from diet-induced weight gain<sup>[149]</sup> most probably because intestinal bacteria are involved in the fermentation of polysaccharides to monosaccharide and in the metabolism of short chain fatty acids<sup>[150]</sup>. The microbiota can also enhance the lipoprotein lipase activity because it reduces the expression of the fasting-induced adipocyte factor in the intestinal epithelium resulting in enhanced FFA storage in adipocytes<sup>[149]</sup>.

LPS produced by intestinal bacteria constitutes the outer membrane of Gram-negative bacteria and can elicit an immune response acting as an endotoxin. LPS may also have a role in the development of obesity, low-grade inflammation and insulin resistance<sup>[151]</sup>. An elegant study by Cani *et al.*<sup>[152]</sup> noticed that high-fat diet induces LPS production in mice and probably its abnormal absorption through the intestinal epithelium may be fat-dependent. The same study has evidenced that endotoxemia induces weight gain, intrahepatic triglyceride accumulation and hepatic insulin resistance, leading to increased expression of TLR4 and proinflammatory cytokines (TNF- $\alpha$ , IL-6, IL-1 and PAI1) in muscle, adipose tissue and liver.

The correlation between intestinal dysbiosis and lipid accumulation in the liver is evidenced by recent research by de Wit *et al.*<sup>[153]</sup>: in mice, a diet with high concentration of palm oil induces higher weight gain and liver triglyceride concentration, reduces microbial diversity and increases *Firmicutes/Bacteroidetes* ratio compared to one high in poly-

unsaturated fatty acids. The fecal microbiota of women following a choline-deficient diet, which induces steatosis, varies during choline depletion and correlates with changes in liver fat concentration, showing modifications in *Gammaproteobacteria* and *Erysipelotrichi* populations<sup>[154]</sup>.

## IBS

Intestinal dysbiosis is also involved in the development of IBS symptoms. The intestinal microbiota modulates intestinal motility and sensitivity<sup>[155]</sup>. An animal study has observed that oral antibiotic therapy perturbs the intestinal microbiota, reduces *Lactobacilli* and decreases *Bacteroides* and *Enterococci* populations, and affects pain perception and visceromotor responses in the gut. The myoelectrical activity in the gut is also altered in germ-free animals and it reversed after colonization<sup>[156]</sup>. The supernatant made from *Escherichia coli* Nissle 1917 stimulates smooth muscle cells and enhances colonic contractility<sup>[157]</sup>, and also *Lactobacillus rhamnosus* GG has a dose- and time-dependent effect on the acetylcholine-stimulated contraction of human colonic muscle cells<sup>[158]</sup>. *Lactobacillus rhamnosus* also has a protective role in pain prevention in animal models<sup>[159]</sup>.

The intestinal bacterial population inhabits a complex environment and its composition varies throughout the gut. It is necessary to distinguish at least three different types of microbiota evaluated in different studies: the luminal microbiota, within the intestinal lumen; the mucosal microbiota that adheres to the intestinal wall; and the fecal microbiota, excreted in feces. In IBS subjects, studies on fecal microbiota have found increased facultative and anaerobic bacteria (as *Escherichia coli* and *Clostridium*) and decreased *Lactobacilli* and *Bifidobacteria*<sup>[160,161]</sup>. Later studies used molecular techniques because most bacterial species in the gut are not cultivable; a recent report of the Rome foundation reviewed principal results<sup>[162]</sup>. The majority of reports have studied fecal microbiota while only a few are focused on the mucosal flora. Furthermore, different molecular techniques are carried out and other limitations may explain that data shown are often contradictory or inconsistent. Moreover, the evidence that SIBO is frequently found in IBS subjects<sup>[163]</sup>, especially in diarrhoea-predominant IBS (D-IBS)<sup>[164]</sup>, and that IBS can develop following infective gastroenteritis (PI-IBS)<sup>[165]</sup> confirms the role of gut dysbiosis in the IBS pathogenesis.

## INTESTINAL PERMEABILITY

A single layer of cells composes the intestinal epithelium, a selective filter and barrier for exogenous substances and water<sup>[129]</sup>. The ways to pass the epithelial layer are mainly two: transcellular and paracellular<sup>[166]</sup>.

The regulation of the paracellular pathway is mainly due to complex structures localized at the apical-lateral and along the lateral membrane between the cells of the intestinal epithelium: desmosomes, adherent junctions and tight junctions (TJs)<sup>[167]</sup>.

TJs regulate selective paracellular ionic solute transport, prevent the passage of luminal antigens, micro-

organisms and toxins, but also regulate the tropism of enterocytes<sup>[168]</sup>. TJs are so called “kissing points”, fusion points where there is no space between two enterocytes<sup>[166]</sup>, and are formed by different transmembrane proteins: tricellulin, occludin, claudins and junctional adhesion molecules, which seal together adjacent cells and cytoskeleton<sup>[169]</sup>.

Several stimuli can modulate the intestinal permeability, but bacterial toxins *inter alia* are able to modify the localization of TJ proteins directly<sup>[170]</sup> or *via* the release of proinflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ <sup>[171]</sup> and IL-6<sup>[172]</sup> that *per se* can reduce the expression of zonula occludens-1 (ZO-1), occludin and claudin.

## NAFLD and NASH

In a recent review, Ilan<sup>[151]</sup> have focused on the role of bacterial translocation in NASH. The bacterial translocation is intimately connected with liver damage from the first step of lipid accumulation in the liver to the development of steatohepatitis, passing through the activation of the innate immune system and mitochondrial dysfunction.

Many animal and human studies have focused on the microbial dysbiosis in NAFLD and to date the endotoxemia, subsequent to bacterial translocation from the gut to the liver through the venous portal system, is an important factor in the development of NASH<sup>[173]</sup>. The mechanisms that lead up to endotoxemia are bacterial overgrowth and impaired intestinal barrier. Sabat  *et al*<sup>[174]</sup>, and previously Wigg *et al*<sup>[175]</sup>, have pointed out that obese subjects have an increased prevalence of SIBO and this condition correlates with severe hepatic steatosis.

Obese mice have a modified distribution of occludin and ZO-1 in the intestinal mucosa in combination with a lower intestinal resistance and higher circulating levels of inflammatory cytokines and portal endotoxemia<sup>[82]</sup>. Similar results are found in mice with fructose-induced steatosis: treatment with metformin leads to a decrease in hepatic triglyceride accumulation and plasma alanine-aminotransferase levels and protection against the loss of the TJ proteins occludin and ZO-1 in the duodenum<sup>[176]</sup>.

In humans, an immunohistochemical analysis of duodenal expression of ZO-1 performed by Miele *et al*<sup>[177]</sup> has highlighted that subjects with biopsy-proven NAFLD have increased gut permeability and high prevalence of SIBO, and that both correlate with the severity of steatosis. Also, NASH subjects have a higher prevalence of SIBO, related to enhanced expression of TLR-4 and release of IL-8<sup>[178]</sup>. The presence of endotoxins in portal blood is found also in cirrhotic patients and is related to an impaired intestinal barrier function<sup>[179]</sup>. Non-cirrhotic NAFLD subjects have increased LPS<sup>[180]</sup> and LPS-binding protein serum levels<sup>[181]</sup>. Probiotic treatment of obese mice leads to a lower intestinal permeability and improved TJ function, a lower plasma LPS and cytokine concentration and a decreased hepatic expression of inflammatory and oxidative stress markers<sup>[182]</sup>. Recently, the association between metabolic syndrome, gut micro-

biota dysregulation and impaired intestinal barrier has been further confirmed in an animal model where dietary obese rats show reduced expression of ZO-1 in the gut and higher TNF- $\alpha$  levels in combination with reduced *Lactobacillus* and increased *Oscillibacter* fecal population. Moreover, TNF- $\alpha$  and IL-6 mRNA levels were higher in mesenteric fat<sup>[183]</sup>.

### IBS

The impaired intestinal permeability is not only a key factor in the development of NAFLD and NASH. Other inflammatory gastrointestinal diseases such as Crohn's disease, UC, bacterial infections caused by *Escherichia coli*, *Clostridium difficile* and *Vibrio cholera*, anti-inflammatory agents associated enteritis and IBS are involved. *In vivo* studies have observed that IBS patients have an impaired intestinal barrier function<sup>[87,90]</sup>. Nevertheless, it is likely that these findings are specific only to D-IBS and PI-IBS subjects and in other IBS subtypes similar results are not found<sup>[87,184]</sup>.

In IBS, intestinal dysbiosis is an important factor participating in damaging the intestinal barrier through the activation of the immune system<sup>[185]</sup> even though another possible cause of impaired intestinal barrier is the exposure to chronic stress. In healthy animals and humans, acute or chronic stress enhances the intestinal permeability to water and also to macromolecules, and IBS subjects are more sensitive to physical and mental stressors compared to healthy subjects<sup>[110]</sup>.

It has been explicated that in IBS subjects there is a low grade inflammation in the gut. Mast cells and T lymphocytes represent the majority of intestinal inflammatory infiltrate and mast cells are also involved in the regulation of motor and visceral responses in the intestine<sup>[19,21,88]</sup>.

The intestinal permeability is controlled by mast cells, *via* histamine, serotonin 5-hydroxytryptamine (5-HT) and protease production<sup>[21]</sup>. Proteases are markedly increased in the mucosa of IBS subjects<sup>[18,186]</sup> and supernatants rich in proteases from D-IBS subjects are able to evoke epithelial dysfunction and allodynia in healthy mice<sup>[20]</sup>. In addition, colonic soluble mediators in supernatants from IBS subjects are able to reproduce permeability alterations in Caco-2 cells and decrease ZO-1 expression<sup>[22]</sup>. A recent study by Martínez *et al.*<sup>[187]</sup> confirms this hypothesis because it has been demonstrated that activated mast cells induce the downregulation of ZO-1 in intestinal epithelium.

Another class of TJ proteins, claudins, is involved too; in fact, claudin-1 and claudin-4 levels are decreased in the small and large intestine of D-IBS patients, whereas claudin-1 and claudin-3 were elevated in constipation-predominant IBS (C-IBS) patients<sup>[188]</sup>.

## INTESTINAL MOTILITY

Intestinal motor and sensory functions are influenced by the immune system to activate a mechanism of defense from noxious agents in the intestinal lumen<sup>[189]</sup>.

Mice infected with *Trichinella spiralis* develop muscle

hyper-contractility in the gut<sup>[190]</sup> but these effects disappear in animal models of athymic and CD4<sup>+</sup> cell-deficient mice<sup>[191]</sup>, encouraging the hypothesis of a role for the immune system and inflammation in intestinal motor functions. Th2 cytokine production was associated with enhanced motor functions and appropriate helminthic elimination. On the other hand, the response with a reduced intestinal motility of Th1, but interestingly also of Th17 cells, seems to be involved in small intestine motor functions. In this setting, specifically IL-17 induces smooth muscle cell contraction<sup>[192]</sup>.

Among Th2 cytokines, IL-13 is secreted by CD4<sup>+</sup> cells and by many other immune cellular types of innate immunity, as the so called "innate helper cells", which can be found normally in the gut and in blood. IL-13 has, in low concentrations, regulatory effects, increasing IL-10 and decreasing IL-17 levels, but, when up-regulated, it leads to inflammatory modifications and hyper-contractility of smooth muscle in the gut<sup>[193]</sup>.

In agreement with these findings, the production of 5-HT, one of the most important neurotransmitters of intestinal motility<sup>[194]</sup>, is also influenced by immune response and cytokine production and its secretion seems to be enhanced by Th2 and reduced by Th1 response<sup>[195]</sup>. 5-HT is synthesized and secreted by enterochromaffin cells (EC) and acts on receptors located on the processes of intrinsic and extrinsic primary afferent neurons in the lamina propria of the gut to initiate peristaltic and secretory reflexes<sup>[196]</sup>. The 5-HT transporter (SERT) is the physiological inhibitor, it is expressed by enterocytes and removes 5-HT from the intestinal space by internalizing it<sup>[197]</sup>.

### Obesity and NAFLD

In high-fat diet fed mice a slower gastric emptying was found, as well as modified intestinal hormone production: higher plasma leptin and cholecystokinin (CCK) concentrations and lower plasma ghrelin levels were found<sup>[198]</sup>. Covasa *et al.*<sup>[199]</sup> have shown that in high-fat diet fed mice there is a reduction in CCK-induced and oleate-induced inhibition of gastric motility.

In obese rats, after Roux-en-Y gastric bypass, an increase in peptide YY and a decrease in ghrelin concentrations occurred. These hormonal modifications may contribute to weight loss by decreasing the food intake and slowing the gastric emptying and transit time<sup>[200]</sup>.

A recent study by Hyland *et al.*<sup>[201]</sup> confirms the presence of an impaired intestinal motility, a modified submucosal nerve function and a decreased electrogenic glucose transport in obese rats. The author hypothesizes that the loss of motor control may lead to an altered host defense and intestinal dysbiosis, and the adapted glucose transport may be a control mechanism in the restriction of nutrient absorption.

Obese subjects have an accelerated esophageal and gastric motility and impaired gastrointestinal hormone secretion<sup>[202,203]</sup>. Vazquez Roque *et al.*<sup>[204]</sup> have detected a lower postprandial gastric volume in obese subjects. A recent report disputes their data: in newborns, fasting

and postprandial gallbladder volumes and gastric emptying were similar between obese and lean subjects, but in obese pre-adolescents, and even more in adults, a larger fasting gallbladder volume with slower postprandial gastric emptying was found<sup>[205]</sup>.

Small and large intestinal motility are also involved, as reported by Xing *et al.*<sup>[206]</sup>. As we see above, SIBO is most frequently viewed in obese subjects and it has been associated with an altered pattern of migrating motor complexes (MMC) in the small intestine<sup>[207]</sup>.

The role of intestinal dysmotility in liver cirrhosis is confirmed by numerous data<sup>[208]</sup>. *Vice versa*, in NAFLD, only a few studies have focused on impaired intestinal motility, although obesity, which is one of the most important etiological factors of NAFLD, is strictly related to impaired intestinal motility. Initial studies have found that NAFLD<sup>[209]</sup> and non-alcoholic cirrhosis<sup>[210]</sup> subjects have a prolonged orocecal transit time.

Interestingly, an up-to-date study correlates 5-HT<sub>3</sub> antagonists to reduced endotoxin levels in the portal system, attenuated liver fat content, inflammation, and cell necrosis, improved TNF- $\alpha$  levels and increased TJ expression in the duodenum of obese, leptin-deficient mice<sup>[211]</sup>. The same group has confirmed these data and has found that SERT deficiency causes hepatic steatosis and impaired intestinal permeability<sup>[212]</sup>. These findings suggest that obesity, and consequently NAFLD, are affected by impaired gut motility and most probably the impaired intestinal barrier, the gut inflammation and also neuronal signaling are key points in their maintenance.

## IBS

IBS subjects frequently report upper gastrointestinal symptoms such as functional dyspepsia<sup>[213]</sup>. Impaired lower esophageal motility and delayed gastric emptying are frequently viewed<sup>[214]</sup> and should be related to small-bowel dysmotility<sup>[215]</sup>.

Many studies have focused attention on the small intestine and large intestine gut dysmotility in IBS subjects. As reviewed elsewhere, studies on MMCs and clustered activity as well as intestinal transit for the small intestine and on myoelectrical activity, intraluminal pressure recordings and transit for the large intestine confirm this hypothesis<sup>[216]</sup>.

In the small intestine of IBS subjects, alterations in the periodicity of MMCs are found<sup>[217]</sup>. Kellow *et al.*<sup>[218,219]</sup> have demonstrated that MMCs have a shorter periodicity in D-IBS, whereas in C-IBS this is longer.

EC cell numbers in the intestinal wall are increased<sup>[220,221]</sup> and postprandial 5-HT levels are increased in platelet-poor plasma<sup>[222]</sup> of IBS subjects, especially in PI-IBS. 5-HT signaling is involved in the pathogenesis of intestinal dysmotility and hypersensitivity; indeed 5-HT modulators are used in IBS therapy<sup>[223]</sup>. 5-HT<sub>4</sub> agonists accelerate colonic transit and are useful in constipation unresponsive to laxative treatment, while 5-HT<sub>3</sub> antagonists inhibit colonic secretion and motility, and visceral sensation, and for this reason are used in D-IBS.

Moses *et al.*<sup>[224]</sup> have found that SERT was less expressed in C-IBS and UC colonic biopsy specimens. Camilleri *et al.*<sup>[225]</sup> have shown that SERT polymorphisms may influence colonic motility in patients with D-IBS and may influence the response to a 5-HT<sub>3</sub> antagonist.

In the colon of IBS subjects activated mast cells in proximity to mucosal innervations may contribute to pain perception<sup>[93]</sup> and are correlated with 5-HT release by intestinal EC cells<sup>[226]</sup>. Interestingly, Mizutani *et al.*<sup>[123]</sup> have observed that in an animal model of IBS, muscle hyper-contraction is related to an increased Th2 cytokine profile (IL-4 and IL-13). Even if these data confirm the role of immune activation in gut motility alteration, it is mandatory to observe that in IBS, and especially in D-IBS and PI-IBS, there is an enhanced gut motor activity even though these IBS subtypes are often related to a Th1 cytokine profile, at least in peripheral blood or in PBMCs. However, there are no reports on the possible role of IL-17 and Th17 in IBS; meanwhile, IL-13 production by PBMCs is higher compared to controls<sup>[124]</sup>.

Recent studies have shown that bacterial products may regulate gastrointestinal motor functions<sup>[227,228]</sup>, but intestinal motility may also influence the gut microbiota composition<sup>[229]</sup>. Pimentel *et al.*<sup>[230]</sup> for the first time demonstrated that the impaired intestinal motility may be related to SIBO in IBS subjects, but subsequent contrasting data have questioned this theory<sup>[163]</sup>. Moreover, as has been described before, IBS and NAFLD are characterized by an intestinal dysbiosis and only a proportion of subjects meet diagnostic criteria for SIBO.

## CNS INVOLVEMENT

A recent review by Capuron *et al.*<sup>[231]</sup> has focused on how the immune system can affect the CNS and contribute to the development of neuropsychiatric disorders such as depression, with particular relevance to cytokine signaling. Cytokines are involved in production, function and reuptake of several neurotransmitters, such as 5-HT. They affect the hypothalamic-pituitary-adrenal (HPA) axis and can modify the neuronal architecture, neuronal plasticity and aging, and neuronal circuits in CNS.

As previously described, 5-HT is an important neurotransmitter of the enteric nervous system (ENS) but it is also fundamental to CNS functioning. 5-HT, produced from tryptophan, plays a major role in the modulation of brain-gut axis<sup>[232]</sup>. The brain-gut axis is constituted peripherally of ENS communicating with the gut wall and centrally with the CNS and HPA axis<sup>[233]</sup>. The gastrointestinal system and the brain communicate in bi-directional mode, both of them influencing each other (the so called top-down and bottom-up model developed in functional GI disorder studies)<sup>[234]</sup>. The HPA axis is composed of corticotropin-releasing hormone (CRH), produced in the hypothalamic para-ventricular nucleus, which stimulates adrenocorticotropin (ACTH) production in the anterior pituitary gland that in turn induces the adrenal cortex to produce cortisol in response to various stressors<sup>[235]</sup>.

In animal and human models the turnover of 5-HT in the brain is altered by acute and chronic exposure to pro-inflammatory cytokines<sup>[236,237]</sup>.

Cytokines stimulate CRH, ACTH and cortisol production and in chronic states influence the diurnal cortisol curve because they stimulate inflammatory signaling that reduces glucocorticoid receptor functions and expression leading to decreased responsiveness to glucocorticoids.

### Obesity and NAFLD

Recently, animal studies have shown that in the hippocampus and cortex of high-fat fed mice there is increased production of inflammatory products<sup>[238,239]</sup> and systemic inflammation is also related to cognitive dysfunctions<sup>[240,241]</sup>. Depression and depressed serotonergic state are strictly related to metabolic syndrome and obesity<sup>[242,243]</sup>. Tarantino *et al.*<sup>[244]</sup> have studied urinary 5-hydroxy-3-indoleacetic acid, a 5-HT metabolite, in depressed and obese/overweight subjects and have found that it negatively correlates with dysthymia and depression status.

Alteration in the HPA axis is well established in obese patients and chronic stress with hyper-alimentation is an important factor in its development<sup>[245]</sup>. Although there are contrasting data on urinary free cortisol (UFC) in obese subjects, a recent study has evidenced in NAFLD subjects increased UFC and cortisol serum concentrations after dexamethasone suppression, both correlated with hepatic inflammation and fibrosis stage<sup>[246]</sup>. Moreover, in a human model, cortisol clearance is increased in NAFLD subjects and is correlated with insulin sensitivity<sup>[247]</sup>. Peripherally, cytokines such as TNF- $\alpha$  and leptin stimulate 11 $\beta$ -HSD1, an enzyme required for the activation of cortisone to cortisol<sup>[248]</sup>. Also leptin and ghrelin increased levels are related to HPA axis dysregulation in obese subjects<sup>[245]</sup>.

Finally, early life stress predisposes to overweight and insulin resistance, at least in animal models<sup>[249]</sup>.

### IBS

Hypersensitivity and brain alterations, investigated with different study methods, have been found in the last 15 years in IBS subjects; and, despite often contradictory data, there is strong evidence of dysregulation in pain and other stimuli perception<sup>[250]</sup>. Moreover, mood disorders (depression, anxiety) and other psychiatric disorders (eating disorders, posttraumatic stress syndrome, panic attack, *etc.*) are frequent, evidencing the role of gut-brain dysfunction in these patients<sup>[107]</sup>.

As has been mentioned above, the majority of reports on 5-HT in IBS have studied its intestinal production; meanwhile, few are focused on its systemic production. Clarke *et al.*<sup>[251]</sup> have found that IBS subjects degrade tryptophan more *via* the kynurenine pathway, an alternative metabolic way producing neurotransmitters other than 5-HT. Subsequently, the same group has found that kinurein from blood of IBS subjects can influence TLR expression<sup>[252]</sup> in an *in vitro* model.

The main evidence on HPA dysregulation in IBS<sup>[250]</sup> is

the following: CRH and ACTH stimulate colonic secretion, intestinal motility, visceral sensitivity and anxiety. Principal brain regions influenced by HPA axis are the amygdala and hippocampus. In IBS there are increased HPA axis responses to stressors such as meals, hormonal stimuli, and mental stress compared to controls. Fatigue and depression are associated with increased mast cell counts in the colonic mucosa of IBS subjects, confirming the role of gut-brain dysfunction in IBS<sup>[253]</sup>. Indeed, a key question still unresolved is whether the SNC dysfunction is the *primum movens* of the gut inflammation and consequently the visceral hypersensitivity and dysmotility in IBS or whether the gut inflammation represents the main cause of subsequent SNC and systemic disorder.

## UNANSWERED QUESTIONS

Could weight loss ameliorate IBS symptoms by influencing intestinal microbiota? Is there a relationship between NAFLD severity and IBS symptoms? Could patients suffering from IBS be at major risk to develop NASH? Are circulating levels of inflammatory cytokines overlapping in IBS subjects and NAFLD? Could intestinal dysbiosis affect CVD risk *via* NAFLD<sup>[254]</sup>?

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## Current status in the treatment options for esophageal achalasia

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### Abstract

Recent advances in the treatment of achalasia include the use of high-resolution manometry to predict the outcome of patients and the introduction of peroral endoscopic myotomy (POEM). The first multicenter randomized, controlled, 2-year follow-up study conducted by the European Achalasia Trial group indicated that laparoscopic Heller myotomy (LHM) was not superior to

pneumatic dilations (PD). Publications on the long-term success of laparoscopic surgery continue to emerge. In addition, laparoscopic single-site surgery is applicable to advanced laparoscopic operations such as LHM and anterior fundoplication. The optimal treatment option is an ongoing matter of debate. In this review, we provide an update of the current progress in the treatment of esophageal achalasia. Unless new conclusive data prove otherwise, LHM is considered the most durable treatment for achalasia at the expense of increased reflux-associated complications. However, PD is the first choice for non-surgical treatment and is more cost-effective. Repeated PD according to an "on-demand" strategy based on symptom recurrence can achieve long-term remission. Decision making should be based on clinical evidence that identifies a subcategory of patients who would benefit from specific treatment options. POEM has shown promise but its long-term efficacy and safety need to be assessed further.

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**Key words:** Esophageal achalasia; Endoscopic pneumatic dilations; Botulinum injection; Peroral endoscopic myotomy; Minimally invasive Heller myotomy

**Core tip:** Recent progress in esophageal achalasia includes the use of high-resolution manometry to predict the outcome, the introduction of peroral endoscopic myotomy (POEM). The best current treatment option is an ongoing matter of debate. Unless there are more new conclusive data to prove otherwise, laparoscopic Heller myotomy is the most durable treatment for achalasia at the expense of reflux complications. However, pneumatic dilation (PD) is the first choice for non-surgical treatment and is more cost-effective. Repeated PD according to an "on-demand" strategy based on symptom recurrence can achieve long-term remission. POEM is optimistic but needs more long-term efficacy and safety reports.

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## INTRODUCTION

Achalasia is one of the primary motility dysfunctions of the esophagus that affects both sexes and all races equally<sup>[1,2]</sup>. The selective loss of inhibitory neurons of the myenteric plexus, which produces vasoactive intestinal polypeptide, nitric oxide (NO), and inflammatory infiltrate, is responsible for abnormal lower esophageal sphincter (LES) dysfunction. This results in unopposed excitation of the LES, and dysfunction or failure of the LES to relax in response to each swallow<sup>[3]</sup>. Dysphagia for both liquid and solid foods is the most common symptom. Food regurgitation is one of the main associated problems, causing pulmonary complications such as chronic cough and aspiration pneumonia. Gradual weight loss usually occurs as a result.

Achalasia is diagnosed on the basis of tests such as barium esophagography, esophageal manometry, and endoscopy. Pseudoachalasia has to be ruled out by performing endoscopic ultrasound, or computed tomography<sup>[4]</sup>. A classic “bird-beak” of the gastroesophageal junction, with atonia and a dilated esophageal body detected by barium ingestion and fluoroscopy, are the typical radiological signs. Manometry is still the standard diagnostic test for achalasia. Conventional manometry must at least meet the criteria of absent or abnormal swallowing relaxation of the LES, and the absence of peristalsis in the esophageal body. However, the sensitivities of these traditional studies have been challenged by the recent emergence of advanced techniques for the diagnosis of esophageal achalasia such as the use of high-resolution manometry (HRM) and the addition of pressure topography plotting<sup>[5]</sup>. Together, these technologies are also called high-resolution esophageal pressure topography<sup>[6]</sup>. HRM with pressure topography plotting is capable of identifying impaired esophagogastric junction relaxation and subcategorize achalasia into three clinically relevant subtypes based on the contractile function of the esophageal body according to the Chicago classification<sup>[6]</sup>. Type I (classic achalasia) refers to patients with no significant pressurization within the esophageal body and impaired LES relaxation (Figure 1A). Water swallows cause rapid pan-esophageal pressurization, which may exceed LES pressure, causing the esophagus to empty for Type II disease (achalasia with compression) (Figure 1B). Type III achalasia, also known as spastic achalasia, is usually associated with rapidly propagated pressurization attributable to an abnormal lumen obliterating contraction (Figure 1C).

Recent advances in the treatment of achalasia include the use of HRM to predict patient outcome, the

introduction of peroral endoscopic myotomy (POEM), and laparoendoscopic single-site Heller myotomy with anterior fundoplication. Contributing to the ongoing debate on the superiority of pneumatic dilation (PD) vs laparoscopic Heller myotomy (LHM), the first multicenter, randomized, controlled, 2-year follow-up study conducted by the European Achalasia Trial group indicated that LHM was not superior to PD<sup>[7]</sup>. Nevertheless, publications on the long-term success of laparoscopic surgery continue to emerge. This review seeks to address this issue and provide an update on the current progress in the treatment of achalasia. Current available treatment modalities include relaxing the LES and relieving the esophageal obstruction<sup>[2]</sup>. The durability of a successful treatment, complication rates, and cost-benefit are the primary concerns.

## TREATMENT OF ESOPHAGEAL ACHALASIA

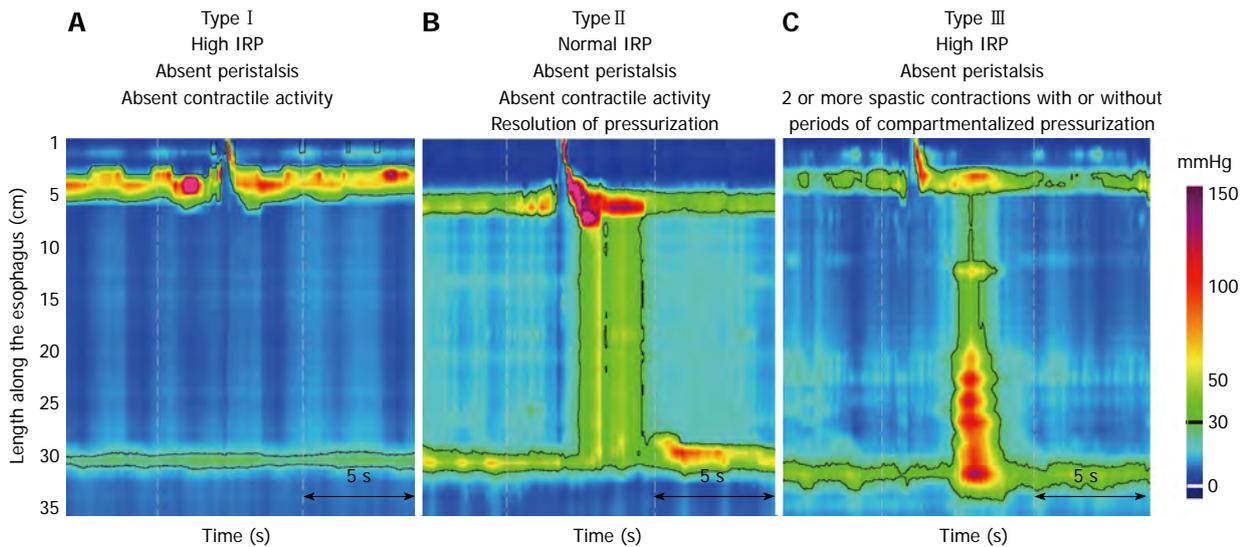
### *Pharmacological management*

Pharmacological management usually plays a minor role in the treatment of esophageal achalasia. Smooth muscle relaxation is partly effective for the reduction of LES pressure<sup>[8]</sup>. NO concentration in smooth muscle cells is increased by medication such as nitrates that increase cyclic GMP levels. Calcium antagonists block calcium entry and hence esophageal muscle contraction. When combined, these drugs can reduce LES pressure and ultimately relieve dysphagia but the efficacy is usually unsatisfactory and incomplete. Furthermore, side effects such as headache, dizziness, and pedal edema are important concerns, and they can be intolerable. These effects are similar to those with other drugs such as sildenafil<sup>[9]</sup>.

### *Endoscopic treatment*

Traditional endoscopic treatments for achalasia include injection of botulinum toxin and PD. Recently, a novel endoscopic technique, POEM, has been introduced and tested by gastroenterologists.

**Botulinum toxin injection:** Botulinum toxin is a biological toxin derived from *Clostridium botulinum* that causes paralysis of both voluntary and involuntary muscles<sup>[10]</sup>. It mainly acts at the terminal nerve endings of myoneural junctions by preventing the release of acetylcholine from vesicles, causing chemical denervation that can persist for several months. Botulinum toxin injection (BTI) is a treatment option for achalasia, and it is associated with a wide safety range and fewer complications<sup>[11]</sup>. Local injection of the toxin into the LES of patients with achalasia lowers sphincter tone, and the patient becomes asymptomatic. This treatment is reported to have excellent immediate responses (success rates > 90%)<sup>[11]</sup>. BTI is associated with a significant improvement in all objective tests of esophageal function, such as decreased LES pressure, increased esophageal diameter, and improvement of transit time by scintigraphy. Complications of BTI



**Figure 1** High-resolution manometry with pressure topography plotting classified achalasia into three subtypes. A: Type I (classic achalasia) refers to patients with absent of peristalsis, no pressurization within the esophageal body, high integrated relaxation pressure (IRP); B: Type II (achalasia with compression) refers to patients with absent of peristalsis, and contractile activity, panesophageal pressurization > 30 mmHg, and high IRP; C: Type III patient (spastic achalasia), associates with absent of peristalsis, and two or more spastic contractions with or without periods of compartmentalized pressurization and a high IRP.

therapy for achalasia are minor, with approximately 25% of patients presenting with transient chest pain and < 5% complaining of reflux symptoms. The dosage used is too small to induce serious adverse effects such as generalized paralysis. The main drawback of BTI is its short duration of effect, which lasts only 6-9 mo in most patients. Based on the number of injections required, the treatment costs are 50% higher than those of PD. Success rates have been reported to be highest among elderly patients and in patients with an LES pressure not exceeding the upper normal level prior to treatment<sup>[12]</sup>. Therefore, it is currently recommended to treat only elderly and high-risk patients with concomitant comorbid diseases.

**PD:** In this simple forceful bougie dilation method, considerable stretching strength is required for the dilation to result in an effective mechanical tear in the muscle fibers of the LES. The most commonly used dilator is the Rigiflex dilator with a fully inflated diameter that is usually  $\geq 3$  cm to achieve a satisfactory result, and is able to achieve maximal pressure. This procedure can be guided using fluoroscopy<sup>[13-15]</sup> or endoscopy<sup>[2,16,17]</sup>. The number of dilation sessions and the inflation time needed for a successful dilation vary and are operator dependent. A single dilation session with a bigger dilator may be used in patients presenting with relapse based on symptom scores<sup>[18]</sup>. Progressive PD methods, such as a series of dilations on successive days using a larger dilator, have been proposed<sup>[19]</sup>. Immediate and short-term results have reportedly been good in most series<sup>[20-25]</sup>. However, the first 5-10 years of published follow-up studies have shown that 20%-75% of patients needed a second or even more dilatations<sup>[26,27]</sup>. Large-scale, long-term follow-up investigations reported unfavorable recurrence in fluoroscopy-guided PD patients<sup>[13,27,28]</sup>. Repeat PD according to an

“on-demand” strategy based on symptom recurrence can achieve long-term remission<sup>[24]</sup>. Post-dilation radiographic findings in addition to the symptom-based scoring systems can reliably predict clinical remission and indicate the need for further treatment in patients with poor esophageal clearance after dilation to avoid progression to sigmoid type achalasia<sup>[29,30]</sup>.

Complications caused by PD are uncommon. The most severe complication is perforation with an incidence of 1%-2% as shown in Table 1. These perforations are usually minor but can be hazardous if undetected after PD<sup>[31]</sup>. Reflux symptoms after PD are usually minor and transient, and can be easily controlled with proton-pump inhibitors.

**Self-expanding metallic stents:** A study evaluating the utility of self-expanding, 30-mm metallic stents for achalasia at a single center over a 10-13-year period reported a long-term clinical success rate of > 80%<sup>[32,33]</sup>. No perforations or mortality associated with the treatment were reported, but stent migration occurred in 5% of patients, reflux in 20%, and chest pain in 38.7%. Overall, the authors claimed that self-expanding, 30-mm metallic stents were associated with a better long-term clinical efficacy in the treatment of patients with achalasia as compared with treatment with PD.

**POEM:** This novel endoscopic esophagomyotomy method for the treatment of achalasia was first reported by Pasricha *et al*<sup>[34]</sup> in porcine models and then by Inoue *et al*<sup>[35]</sup> in humans. POEM is performed by dissection and division of the inner circular muscle layer of the esophagus through a submucosal tunnel created endoscopically by a small proximal opening of the esophageal mucosa. When compared with surgical myotomy, POEM can accomplish a longer myotomy. Extending the length of the myotomy

**Table 1** Cumulative effectiveness of pneumatic dilations for treatment of achalasia by using low compliance “Rigiflex” dilators

No.	Type of dilator (size, cm)	Improvement excellent/good	Mean follow-up (yr)	Complication perforation	Ref.
125	3.0-4.0	50%	12.00%	0.01%	[27]
54	BMD	36%-40%	13.80%	0.02%	[13]
262	3.0-3.5	60%	4.50%	1.00%	[14]
66	3.0-4.0	79%	4.60%	5.00%	[15]
39	3.0-4.0	58.3%-78.0%	9.30%	5.40%	[24]
50	3.0-4.0	67%-83%	2.70%	0.00%	[25]
106	3.0-4.0	28%-62%	3.20%	2.80%	[28]
209	3.0-4.0	72%	5.80%	0.00%	[23]
55	3.0-3.5	74.50%	2.30%	0.00%	[22]
43	3.0-3.5	54%-78%	2.40%	2.30%	[17]
56	3.5	89.3%-92.9%	0.50%	0.00%	[21]
32	3.0	69%-91%	4.50%	3.30%	[20]
1097	3.0-4.0	28.0%-92.9%	0.5%-15.0%	1.0%-2.0%	Total

BMD: Browne-McHardy dilator.

to the thoracic esophagus is difficult for the surgeon, especially in patients with advanced disease and in those with severe fibrosis. Theoretically, the risk of injury to the vagus nerve should be lower with this approach.

Increasing numbers of reports on this technique have been published, and all of them showed good short-term results without serious complications; however, long term follow-up results are necessary<sup>[35-45]</sup>. A recently published prospective, international, multicenter study that aimed to determine the outcomes of 70 patients who underwent POEM at five centers in Europe and North America showed that the percentages of patients with symptom remission at 6 and 12 mo were 89% and 82%, respectively. Zhou *et al.*<sup>[45]</sup> reported that POEM was a promising new treatment for failed Heller myotomy, resulting in short-term symptom relief in > 90% of cases. Nevertheless, POEM can be a challenging and demanding technique even for experienced endoscopists. Although air leak such as that caused by pneumomediastinum, pneumoperitoneum, and air embolism can be prevented by carbon dioxide insufflation, it can be hazardous in cases of purulent mediastinitis. If it occurs, extensive surgical procedures such as esophagectomy may be necessary instead of revisional surgery because of the inflamed and scarred tissue of the plane between the submucosal and muscular layers after the endoluminal approach<sup>[35-41]</sup>.

**Surgical treatment**

Myotomy of the LES is the best treatment modality with satisfactory long-term results at the deleterious cost of a high incidence of postoperative reflux. Although controversy exists as to whether a concomitant antireflux procedure is necessary, minimally invasive LHM with a variety of fundoplication procedures has become the primary approach by many surgeons in the majority of patients with achalasia<sup>[46-49]</sup>. The overall success rates were between 77.0% and 97.2%<sup>[46-57]</sup> (Table 2). However, different surgeons have different opinions on the length of the myotomy. Generally, most surgeons choose a

**Table 2** Cumulative effectiveness of surgical myotomy for achalasia

No.	Type of surgery	Improvement excellent/good	Mean follow-up (yr)	Complication acid reflux	Ref.
52	LHM-Dor operation	92%	4.3	11%	[51]
53	LHM-Dor operation	92%	3.0	9%	[61]
75	LHM-partial fundoplication	84%	5.6	15%	[48]
71	LHM-Dor	85%	6.0	12.70%	[52]
248	LHM+/-Dor	88%	3.4	3%	[55]
211	LHM-Dor	89%	5.3	34%	[56]
161	LHM-Dor	97.20%	4.6	15.70%	[47]
200	LHM-Dor	85%	3.5	28%	[57]
46	LHM-Toupet or Dor	80%	6.4	9%	[46]
505	LHM+/- fundoplication	95%	2.6	16%	[51]
155	LHM-Dor	77%	5.0	27%	[54]
137	LHM-Dor	94.80%	5.4	10.90%	[59]
1860	-	77%-97.2%	2.6-10.9	3%-34%	Total

LHM: Laparoscopic Heller myotomy.

myotomy length of 4-5 cm onto the esophagus and 2-3 cm onto the stomach<sup>[58]</sup>. Another controversial issue among surgeons is whether a concomitant antireflux procedure is necessary. Currently, most surgeons perform minimally invasive LHM with a variety of fundoplication procedures in the majority of patients with achalasia, and partial fundoplications are preferred because 360° fundoplications cause more dysphagia<sup>[46,47,59,60]</sup>. Randomized controlled trials have shown that the addition of an antireflux procedure to a myotomy substantially reduces the postsurgical incidence and severity of pathological reflux<sup>[61,62]</sup>. Recently, laparoendoscopic single-site surgery has proven to be an archetypal shift to more minimally invasive surgery, and is applicable to advanced laparoscopic operations such as LHM and anterior fundoplication<sup>[63]</sup>. Esophagectomy may be needed in patients with recurrent disabling symptoms or severe complications.

Overall, postsurgical complications are rare (< 4%)<sup>[64]</sup>. The major adverse event associated with surgery is severe reflux (3%-34%, Table 2). To minimize the reflux complications, it is generally accepted that a concomitant endoscopic examination during LHM to guide the myotomy and routine fundoplication is clinically necessary with either anterior fundoplication (Dor) or partial posterior fundoplication (Toupet)<sup>[65-67]</sup>. LMH is superior to thoracoscopic procedures because of the shorter operative time and hospital stay<sup>[68]</sup>.

The reported incidence of esophageal perforation in LHM is 5%-10%. However, robotically assisted Heller myotomy (RAHM) is safer than LHM because it decreases the incidence of esophageal perforation to 0%, even in patients who had undergone previous treatment<sup>[69,70]</sup>. RAHM with partial fundoplication using a robotic platform appears to be a more precise and safer operation than laparoscopic myotomy, with improved postoperative quality of

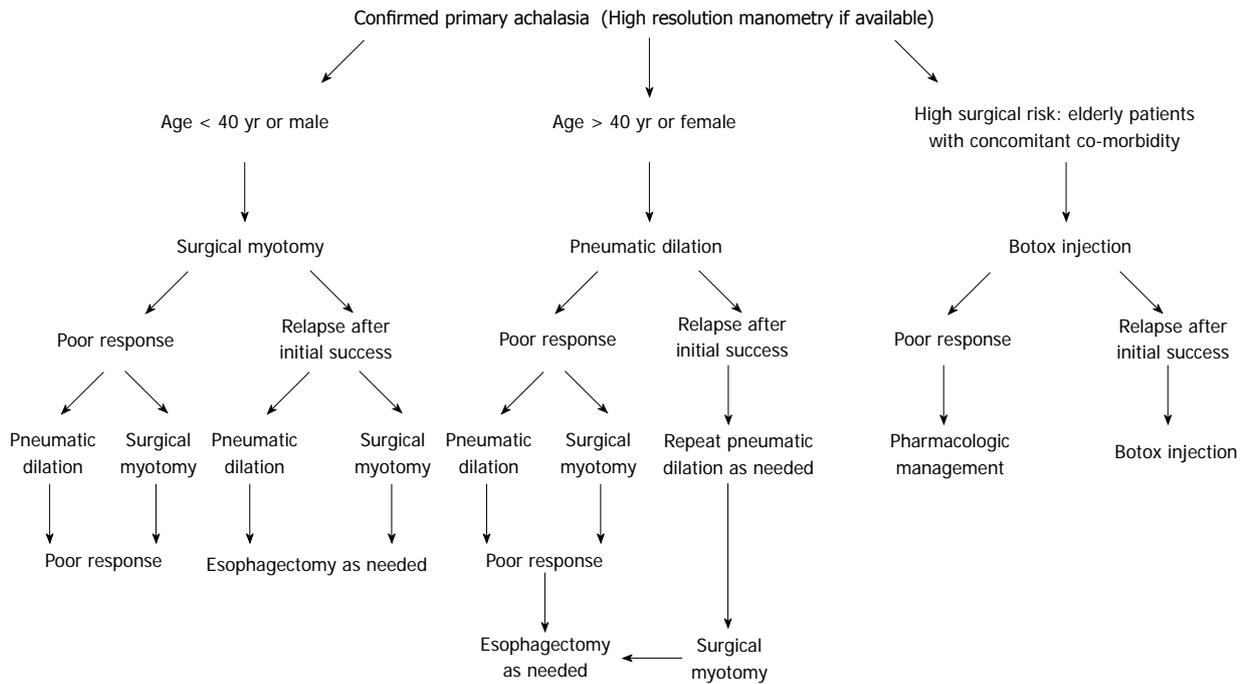


Figure 2 Proposed algorithm for the treatment of esophageal achalasia.

life. In addition, the outcomes of RAHM are slightly better than those of LHM, although the cost is higher<sup>[70]</sup>.

### DECISION MAKING: WHICH IS THE MOST APPROPRIATE TREATMENT?

A proposed algorithm for the selection of the optimal treatment modality for esophageal achalasia is summarized in Figure 2. LHM and PD remain the key treatment options. The short-term efficacy of BTI therapy is similar to that of PD, although it is less effective for sustained symptomatic relief in patients with achalasia in comparison to PD and LHM. BTI is also effective in patients with tortuous megaesophagus and previous failed pneumatic dilatations; however, a high rate of relapse during the first year of follow-up has been reported<sup>[71]</sup>. The selection of BTI should be made with caution in certain patients because some surgeons reported that it increased the risk and difficulty of subsequent LHM<sup>[72]</sup>. Therefore, taking into account the lower durability of BTI therapy, it is a suitable alternative only in the minority of high-risk patients with comorbidity<sup>[2,8]</sup>.

POEM can achieve favorable short-term results comparable to those of any of the above treatment modalities. Moreover, it enables the performance of a longer myotomy, especially in patients with advanced disease and those with severe fibrosis, with a lower risk of injury to the vagus nerve. Validation of the long-term durability and safety of this procedure could make POEM a breakthrough in the treatment of esophageal achalasia. Long-term follow-up of patients who undergo POEM is important to test the durability and safety of the procedure. After all, it is a very technically demanding procedure.

### Surgery vs PD

The choice of LHM as the primary treatment for achalasia or as second-line treatment following the failure of nonsurgical intervention remains a topic of controversy after many decades in clinical practice. Several studies have shown that repeated PD according to an on-demand strategy based on symptom recurrence can increase success rates to levels comparable with LHM<sup>[26,73,74]</sup>. This was supported by the first multicenter, randomized controlled, 2-year follow-up study conducted by the European Achalasia Trial group, which indicated that LHM was not superior to PD<sup>[7]</sup>, and it supports those who are in favor of PD, believing that PD and LHM are equally efficient. This is further supported by advantages of PD such as the fact that it is an outpatient procedure associated with minimal injury, and minimal reflux and bleeding. However, the follow-up duration of this study is not long enough to declare equality between the two procedures. In addition, another disadvantage of PD is that these patients usually require more than one treatment session. Nevertheless, publications on satisfactory long-term success of laparoscopic surgical outcome continue to emerge<sup>[75]</sup>, and patients usually require only one treatment session. Moreover, some surgeons believe that LHM can be more difficult technically following PD but others claim that PD does not hinder future myotomy procedures<sup>[76]</sup>.

Complications and cost-effectiveness, besides the durability of the procedure, are the main concerns for deciding on a treatment option. Perforation of the esophagus occurs in 1%-2% of patients during PD and can be hazardous if left undiscovered<sup>[51]</sup>. Mucosal tears occur in 12% of patients during LHM but can usually be repaired, and the patients recover. However, the main drawback of LHM is the incidence of acid reflux after surgery, which

**Table 3 Summary of the cumulative efficacies and complications of current treatment options of achalasia**

	PD	Surgical myotomy	BTI	POEM
No. of studies	12	12	9	11
No. of patients	1097	1860	315	210
Excellent/good symptom response (range)	28%-92.9%	77%-97.2%	At 1 mo: 79% (64%-93%) At 1 yr: 41% (10%-55%)	82%-100%
Follow up (yr)	0.5-15	2.6-10.9	18 (6-30)	0.1-1
Major complications (range)	1%-2% Perforation	3%-34% Acid reflux	-	0.03% Acid reflux

PD: Pneumatic dilations; BTI: Botulinium toxin injection; POEM: Peroral endoscopic myotomy.

could be long lasting despite partial fundoplication. Reflux can usually be treated with proton-pump inhibitors; however, long term complications of reflux such as stricture, Barrett’s esophagus, and adenocarcinoma, although rare, must be kept in mind. By contrast, symptoms of reflux in post-PD patients are usually mild and transient and can be easily controlled by prescribing proton-pump inhibitors. When considering the cost-effectiveness of treatment strategies for achalasia, LHM has a higher initial cost and PD is the most cost-effective treatment option for adults with achalasia<sup>[77]</sup>. However, LHM can be cost-effective if the durability is > 10 years<sup>[78]</sup>. A recent meta-analysis conducted by Weber *et al*<sup>[79]</sup> showed that both PD and LHM are effective treatment options, but LHM might be more durable.

The experience of the surgeons and gastroenterologist is also an important factor for treatment success. More importantly, the decision should be based on clinical evidence that identifies a subcategory of patients who may benefit from a specific treatment option. In general, unless new conclusive data prove otherwise, LHM is the more durable treatment for achalasia, but PD is the first nonsurgical choice and is more cost-effective. Practically, the correction of failed operations for esophageal achalasia is challenging; however, those operations are also performed at high-volume centers using laparoscopic procedures, and many patients prefer to avoid esophagectomy. However, some researchers have reported adverse effects of repeated dilations, especially the risk of perforations, and this must be considered in the decision making process. LMH is recommended for younger patients (< 40 years), male sex, and those showing pulmonary symptoms and failed response to one or two initial dilations<sup>[2,8,80]</sup>.

**PREDICTORS OF RISK FACTORS FOR RELAPSE AFTER TREATMENT FOR ACHALASIA**

To recognize the risk factors for relapse after treatment is an important issue. It is generally accepted that

young age (< 40 years), male sex, a single dilation session with a 3.0-cm balloon, immediate or 3-mo post-treatment LES pressure > 15 mmHg, poor esophageal emptying on timed barium swallow, and classic achalasia are considered the predicting risk factors for relapse after PD<sup>[2,8,20,21]</sup>. Therefore, both timed barium esophagography and manometry, especially HRM, should be performed at baseline and post-PD, and compared to predict the outcome of patients. The possible impact of the results of HRM on treatment outcome was highlighted in Pandolfino’s landmark study, which showed that Type II achalasia patients were significantly more likely to respond to any therapy [BTI (71%), PD (91%), or LMH (100%)] compared with Type I (56% overall) or Type III (29% overall) patients. Type II achalasia was a predictor of positive treatment response, whereas Type III and pretreatment esophageal dilatation were predictive of a negative treatment response<sup>[81]</sup>. This was confirmed in another study by Pratap *et al*<sup>[82]</sup>, which showed that patients with a Type II achalasia pattern (esophageal pressurization) on HRM were more likely to respond to all therapies such as PD, Heller myotomy, and BTI (70%-100% overall), as compared with Type I ( $\geq$  63.3% overall) and Type III approximately 30% overall) patients. More evidence with larger prospective studies and long-term follow-up results are necessary in the new era of HRM (Table 3).

**FUTURE PERSPECTIVES**

Most existing studies point toward autoimmune mechanisms affecting neurons possibly after an infectious event and an association with certain genetic factors as the possible etiology<sup>[83]</sup>. The identification of an immunomodulatory drug for the treatment of achalasia is a target to achieve in the future. Evidence indicates that transplanting neuronal stem cells could be “a dream come true” achievement in the future<sup>[84]</sup>. Theoretically, if this works, both LES function and peristalsis should recover.

**CONCLUSION**

The debate on PD and LHM is on-going. Unless new conclusive data prove otherwise, LHM is a more durable treatment option for achalasia at the expense of increased reflux complications. However, PD is the first nonsurgical choice and is more cost-effective. Repeated PD according to an on-demand strategy based on symptom recurrence can achieve long-term remission. It is recommended that the decision making should be based on clinical evidence that identifies a subcategory of patients who may benefit from a specific treatment option. POEM is a promising strategy, but more long-term efficacy and safety studies are necessary.

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## Practice guidelines for ultrasound-guided percutaneous microwave ablation for hepatic malignancy

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the most frequent malignancies worldwide, with an increasing number of new cases and deaths every year. Traditional surgery is only suitable for a limited proportion of patients and imaging-guided percutaneous thermal ablation has achieved optimistic results for management of hepatic malignancy. This synopsis outlines the first clinical practice guidelines for ultrasound-guided percutaneous microwave ablation therapy for hepatic malignancy, which was created by a joint task force of the Society of Chinese Interventional Ultrasound. The guidelines aim at standardizing the microwave ablation procedure and therapeutic efficacy assessment, as well as proposing the criteria for the treatment candidates.

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**Key words:** Practice guidelines; Microwave radiation; Catheter ablation; Liver cancer; Ultrasound

**Core tip:** Thermal ablation has undergone rapid development as a minimally invasive procedure, with optimistic results and rapid rehabilitation. This synopsis outlines the first clinical practice guidelines for ultrasound-guided percutaneous microwave ablation therapy for hepatic malignancy, which was created by a joint task force of the Society of Chinese Interventional Ultrasound. The guidelines aim at standardizing the microwave ablation procedure and therapeutic efficacy assessment, as well as proposing the criteria for treatment candidates.

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### Abstract

Primary liver cancer and liver metastases are among

## INTRODUCTION

Primary liver cancer is the sixth most commonly diagnosed cancer worldwide and hepatocellular carcinoma (HCC) accounts for 70%-90% of the total incidence. There were 748300 new liver cancer cases and 695900 cancer deaths worldwide in 2008 and half of the cases and deaths were estimated to occur in China as a result of the high prevalence of chronic viral hepatitis<sup>[1,2]</sup>. Metastases are another common hepatic malignancy. Colorectal liver metastasis is one of most common hepatic metastases. It has been reported that 14.5%-23.0% of colorectal cancer patients have synchronous liver metastases at the time of exploration for their primary tumor and 76.8% eventually develop liver metastases<sup>[3]</sup>. A number of different locoregional therapies for hepatic malignancy have been performed, including surgical resection, percutaneous ethanol injection, microwave ablation (MWA), radiofrequency ablation (RFA), high-intensity-focus ultrasound and transcatheter arterial chemoembolization (TACE). Traditionally, surgical resection is the reference standard for treatment of patients with hepatic malignancy, however, only a small proportion of them have the chance to be candidates because of disease progression, anatomical location, and poor liver function. As an alternative therapy, imaging-guided percutaneous ablation has been widely applied for management of hepatic malignancy, owing to its advantages of minimal invasion, favorable efficacy, and reproducibility<sup>[4-7]</sup>. Among thermoablative techniques, RFA is the most extensively used worldwide. MWA of liver cancer was first adopted in Japan by Saito *et al.*<sup>[8]</sup> and has been widely applied in China over the past two decades<sup>[5,6,9-15]</sup>. Several studies<sup>[16-19]</sup> showed that the local tumor control, complications and long-term survival were equivalent for RFA and MWA in treatment of hepatic malignancy. A recent multicenter study from China documented that 1007 patients with primary liver cancer treated by MWA achieved 1-, 3-, and 5-year survival rates of 91.2%, 72.5%, and 59.8%, respectively<sup>[20]</sup>. For liver metastases, MWA offers a mean 1-, 3- and 5-year survival rate of 73%, 30% and 16%, which represents an advantage over palliative chemotherapy even in patients with extrahepatic disease<sup>[17]</sup>.

## PURPOSE

The purpose of these guidelines is to establish basic clinical practice guidance to assist physicians with: (1) evaluating patients with hepatic malignancy, including primary liver cancer and liver metastases, who may be candidates undergoing percutaneous MWA under ultrasound (US) guidance; (2) providing relevant and updated technical information for performing this treatment; and (3) understanding the consequences of this treatment.

A working group including 44 experts from the Society of Chinese Interventional Ultrasound (SCIU) met in June 2011 to consider the evidence for developing the draft guidelines. Additional meetings were conducted *via*

teleconference. The guidelines were circulated in draft form to the full expert panel for review and approval. In addition, practitioner feedback was obtained from physicians in the province of interventional treatment, and their comments were incorporated into the guidelines. These recommendations represent the panel's attempt to extract practical guidelines from a combination of published evidence and expert opinion where the literature falls short.

## LITERATURE SEARCHES

The expert panel completed the review and analysis of data published since 1990. Computerized literature searches of MEDLINE, EMBASE and the Cochrane Collaboration Library were performed. The searches of the English-language literature from 1990 to June 2011 combined the terms "hepatic neoplasms" and "liver neoplasms", with the MeSH terms "microwaves" and "catheter ablation". The searches were limited to human-only studies and to specific study designs or publication types: randomized clinical trials, meta-analyses, systematic reviews, and major clinical trials in MWA of liver tumors.

## DESCRIPTION OF MWA

### *Mechanism*

MWA refers to all electromagnetic methods of inducing tumor destruction by using devices with frequencies  $\geq 900$  MHz<sup>[21]</sup>. The rotation of dipole molecules accounts for most of the heat generated during MWA<sup>[22,23]</sup>. Water molecules are dipoles with unequal electric charge distribution, and they attempt to reorient continuously at the same rate in the microwave oscillating electric field. Therefore, electromagnetic microwaves heat matter by agitating water molecules in the surrounding tissue, producing friction and heat, thus inducing cellular death *via* coagulation necrosis. Another mechanism responsible for heat generation is ionic polarization, which occurs when ions move in response to the applied electric field of microwaves. Displacement of ions causes collision with other ions, which converts kinetic energy into heat. However, it is a far less important mechanism than dipole rotation in living tissue. Currently, two kinds of frequencies: 915 and 2450 MHz are used for MWA. A frequency of 2450 MHz is more commonly adopted, which is also the frequency used in conventional microwave ovens given optimal heating profiles<sup>[23]</sup>. Microwaves of 915 MHz can penetrate more deeply than 2450 MHz microwaves<sup>[24]</sup>, therefore, the low frequency MWA may theoretically yield larger ablation zones.

### *Technical advantages*

MWA shows the following theoretical technique advantages over RFA. (1) active tissue heating of RFA is limited to a few millimeters surrounding the active electrode, with the remainder of ablation zone relying on the conduction of electricity into the tissue<sup>[22]</sup>. Microwaves use

electromagnetic energy with the much broader field of power density (up to 2 cm surrounding the antenna) to rotate rapidly adjacent polar water molecules to achieve primarily active heating, which can yield a much broader zone of active heating<sup>[21]</sup>; (2) RFA is limited by the increase in impedance with tissue boiling and charring<sup>[22]</sup>, because water vapor and char act as electrical insulators. MWA does not seem to be subject to this limitation. Therefore, temperature > 100 °C is readily achieved<sup>[25]</sup>; (3) Owing to the active heating ability, MWA can achieve higher intratumoral temperatures, larger ablation volumes, and shorter ablation times<sup>[25-28]</sup>. Because the cooling effect of blood flow is most pronounced within the zone of conductive rather than active heating, MWA is less affected by blood-vessel-mediated cooling (the heat-sink effect). These benefits have the potential to allow for a more uniform tumor kill in the ablation zone, both within the targeted zone and perivascular tissue<sup>[28,29]</sup>; (4) MWA allows for simultaneously multiple probe deployment to reduce the duration of therapy and increase the diameter of ablation zone<sup>[21,22,25]</sup>; and (5) MWA does not require the placement of grounding pads and the electrical energy is deployed in the target tissue only, which avoids applied energy loss and skin burns. Moreover, MWA is not contraindicated by the metallic materials like surgical clips or pacemaker.

However, as one of most recent advances in the field of thermoablative technology, MWA has a few limitations: (1) The higher thermal efficiency of MWA may become a double-edged sword to injury easily the adjacent critical tissues because of the tissue surrounding the antenna being rapidly ablated; and (2) Simultaneous deployment of multiple probes of microwave antennae can significantly increase the diameter of the ablation zone, whereas recession of the coagulation zone for the inter-antenna distance may not entirely cover the large tumor and result in incomplete ablation<sup>[30]</sup>.

Apart from theoretical comparison of technical characteristics, in limited comparative clinical trials between MWA and RFA, two ablation techniques achieved similar tumor necrosis effects and survival<sup>[18,19,31,32]</sup>. However, Japanese researchers thought RFA had a tumor control advantage in small liver lesions<sup>[33,34]</sup>. However, randomized controlled trials with large samples and long-term follow-up are lacking and are strongly recommended to provide evidence-based medicine.

### Equipment

All MWA systems are composed of three basic elements: microwave generator, low-loss flexible coaxial cable, and microwave antenna. Microwaves are generated by a magnetron in the generator. Antennae are connected *via* a low-loss coaxial cable to the generator and transmit microwaves from the magnetron into the tissue. Antennae can be classified as three types (dipole, slot, or monopole), based on their physical features and radiation properties<sup>[35]</sup>. Antenna shape includes straight, loop and triaxial. Design of the antenna is crucial to the therapeutic

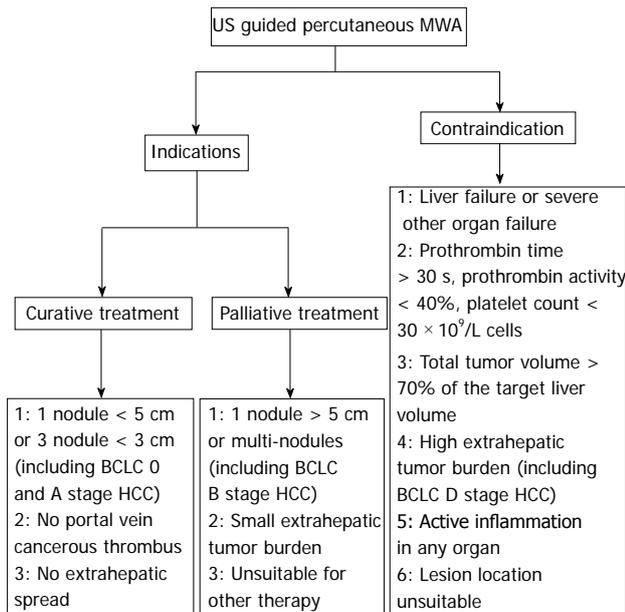
efficacy. Currently, the design has focused largely on needle-like, thin, coaxial-based interstitial antennae<sup>[35-37]</sup>, for the purpose of achieving larger ablation zones and being appropriate for percutaneous use. To prevent over-heating of the shaft, avoid skin injury, and permit further deposition of energy into tissue with low impedance during ablation, cooled-shaft antennae have been developed in recent years. The cooled-shaft antennae have facilitated remarkable progress in obtaining larger ablation zones<sup>[25,38]</sup>. The diameter of the antenna is from 1.6 to 2.8 mm (10-16 G), while the antenna with a diameter of 14-16 G is clinically commonly used.

Some types of commercially available radiofrequency devices contain a thermocouple in the nickel-titanium lateral tine of expandable electrode tip to allow temperature recording during the ablation procedure. The aim of temperature monitoring is to ensure that the maximum energy is applied by using the standard algorithm with the system<sup>[39]</sup>. The microwave machine can also be equipped with a thermal monitoring system that continuously measures temperature in real time during ablation. The thermal monitoring needles are usually classified into thermocouple and thermistor types, with a diameter of 0.7-0.9 mm (20-22 G). The thermal monitoring needle is inserted into the target area through a nonconducting needle trocar for real-time temperature monitoring during ablation under US guidance. The purposes of temperature monitoring include the following: (1) Therapeutic: the temperature monitoring needle is inserted about 5-10 mm away from the tumor margin. Total tumor necrosis is considered to be achieved when the temperature remains at 54 °C for at least 3 min or reaches 60 °C instantly; and (2) Protective: for high-risk localized tumors (< 5 mm from the vital tissues, such as bile duct, gastrointestinal tract, gallbladder, and blood vessels), the real-time temperature of the tumor margin is monitored to ensure that temperature does not reach damaging levels. The temperature cutting off of ablation therapy is set at 54 °C in the patients without a history of prior laparotomy, or 50 °C in patients with a history of laparotomy. The emission of microwaves is reactivated after the temperature decreases to 45 °C, and then in cycles until the entire tumor is completely encompassed by hyperchoic water vapor.

## DIAGNOSIS AND INDICATIONS

### Diagnosis

Pathological diagnosis is necessary for both HCC and metastatic cancer patients. The specific pathological result ensures that the tumor ablated is actually malignant, and tumor differentiation will also provide forceful surveillance guidance for the patients. Furthermore, the metastatic site can be confirmed to guide future chemotherapy and radiotherapy schedules. If the patients need to undergo biopsy to achieve pathological diagnosis, it is preferred to perform intraoperative tumor biopsy before ablation under US guidance. According to several



**Figure 1** Indications and contraindications of ultrasound-guided percutaneous microwave ablation. MWA: Microwave ablation; BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; US: Ultrasound.

reports with large-volume liver cancer patients treated by MWA, the neoplastic seeding as a complication of liver puncture is low risk with a rate of 0.4%-0.6%<sup>[10,40,41]</sup> and is considered generally acceptable. Ablation immediately after biopsy might decrease seeding rate after biopsy and the thermal effect can stop bleeding after biopsy.

If the patient has obtained a histopathological diagnosis during previous treatment, or the tumor location or the patient's condition is not appropriate for the biopsy procedure, a combination of contrast-enhanced US, contrast-enhanced computed tomography (CT) and/or magnetic resonance imaging (MRI) associated with a rising serum tumor marker level is recommended. Contrast-enhanced imaging should include early arterial phase enhancement and be performed to define better the extent and number of primary lesions, vascular anatomy, vessel involvement, tumor involvement, and extrahepatic disease<sup>[42-44]</sup>.

### Indications

Given the complexity of the hepatic malignancy, multi-disciplinary assessment of tumor stage, liver function, and physical status is required for proper therapeutic planning. In general, the indications for MWA are broad (Figure 1). One important application is to treat patients who are not considered surgical candidates. Included in this category are patients with inadequate liver remnant to tolerate resection, tumor multinodularity, unresectable lesions at difficult anatomical locations, or patients who decline resection. Previous MWA was limited to treat small liver tumors, but with the improvement of antennae and treatment strategies, lesions 5-8 cm can also be effectively ablated<sup>[10,45,46]</sup>.

For patients with very early stage and early stage HCC

[based on the Barcelona Clinic Liver Cancer (BCLC) Staging System<sup>[47]</sup>] and limited metastases, MWA should be considered as curative therapy. The inclusion criteria are: (1) a single nodule with a diameter < 5 cm or a maximum of three nodules with a diameter < 3 cm; (2) absence of portal vein cancerous thrombus; or (3) no extrahepatic spread to surrounding lymph nodes, lungs, abdominal organs, or bone.

Palliative treatment criteria for MWA include patients (1) with lesions > 5 cm in diameter or multiple lesions (including BCLC B stage HCC); (2) suffering from a small extrahepatic tumor burden (including part of BCLC C stage HCC); or (3) unsuitable for other modalities and capable of tolerating the MWA procedure.

### Contraindications

Contraindications include patients who have: (1) clinical evidence of liver failure, such as massive ascites or hepatic encephalopathy, or with a trance-like state; (2) severe blood coagulation dysfunction (prothrombin time > 30 s, prothrombin activity < 40%, and platelet count <  $30 \times 10^9/L$  cells); (3) high intrahepatic tumor burden (tumor volume > 70% of the target liver volume or multiple tumor nodules) or high extrahepatic tumor burden (including BCLC D stage HCC); (4) acute or active inflammatory and infectious lesions in any organ; (5) acute or severe chronic renal failure, pulmonary insufficiency or heart dysfunction; and (6) tumor proximity to diaphragm, gastrointestinal tract, gallbladder, pancreas, hepatic hilum and major bile duct or vessels. Successful treatment of the high-risk localized tumor may require adjunctive techniques (*e.g.*, artificial fluid infusion or percutaneous ethanol injection) to prevent off-target heating of adjacent structures during the ablation procedure.

## PATIENT PREPARATION AND DATA REQUIRED

Patients considered for MWA should be accurately evaluated through clinical history, physical examination, laboratory values and performance status. Pre-therapy evaluation of serum liver enzymes, cholinesterase, blood cell count, coagulation, creatinine, and tumor markers such as  $\alpha$ -fetoprotein/carcinoembryonic antigen should be monitored and known before the procedure. The impaired liver function and coagulation status need to be corrected to withstand the ablation procedures. A full pre-ablation imaging work-up (a combination of contrast-enhanced imaging including US, CT or MRI) should be performed to stage, locate the lesions and exclude portal venous thrombosis and metastases accurately (Table 1).

Patients should receive both written and verbal information about the procedure prior to therapy. Informed written consent must be obtained from the patient. Patients should be informed that this therapy is not likely to cure their disease and is a palliative treatment directed at their liver lesions. Patients must be informed of the potential side effects of therapy as well.

**Table 1** Indications and check list for microwave ablation of hepatic malignancy

Curative therapy	Palliative therapy	Check list
Single nodule with a diameter < 5 cm	Lesion > 5 cm in diameter	Histocytologic diagnosis
Maximum of 3 nodules with a diameter < 3 cm	Multiple lesions	US features of nodule (blood, location and size)
Absence of portal vein cancerous thrombus	Suffering from a small extrahepatic tumor burden	CEUS, CT or MRI of liver (lesion number, size, blood and location, portal venous thrombosis)
No extrahepatic spread	Unsuitable for other modalities	Laboratory tests (routine, coagulation function, serum biochemical item and tumor markers)

US: Ultrasound; CEUS: Contrast-enhanced ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging.

## TECHNIQUES

Patients are laid in the supine or oblique position in the interventional US suite. Color Doppler and gray-scale US are performed to choose the safest intercostal or subcostal needle access. Local anesthesia and/or intravenous conscious analgesia-sedation is usually sufficient for the percutaneous approach. Local anesthesia is induced first with 1% lidocaine from the insertion point at the skin to the peritoneum along the US-guided puncture line before inserting the antennae. Then, the skin is pricked with a small lancet, and the antenna is introduced into the chosen area of the tumor. In the multiple-needles procedure two or three prefixed puncture lines are made. Two or three active needle antennae directly connected to the MW generator are inserted into the tumor in parallel 1-2.5 cm apart. After placing all the antennae (breathing cooperation is required from the patient to complete the insertion), venous conscious analgesia-sedation is induced with propofol and ketamine associated with standard hemodynamic monitoring. At each insertion, the tip of the needle is placed in the deepest part of the tumor. Multiple thermal lesions are created along the major axis of the needle antenna by simply withdrawing the needle from the preceding thermal lesion, and reactivating the MW generator. If necessary, due to tumor size, multiple overlapping ablations are usually needed to envelope the entire tumor with a safety margin. In general, the microwave energy application is set at 50-80 W for 5-10 min in a session.

Size of the ablation zone can be roughly judged by an expanding hyperechoic area arising during the procedure. For accurate assessment of the treatment efficacy, the thermal monitoring system attached to the MW generator can be used during MWA. One to three thermocouples are placed at different sites 5-10 mm outside the tumor. The thermocouple can be introduced into the parenchyma through an 18 G, 70-mm long, nonconducting needle trocar. If the measured temperature does not

reach 60 °C by the end of treatment and does not remain at 54 °C for at least 3 min, the treatment is prolonged until the desired temperature is reached. Overheating can also be avoided by thermal monitoring, thus decreasing the incidence of complications. In recent years, contrast-enhanced US has been used for immediate assessment of technical success which is performed 10-15 min after MWA<sup>[48]</sup>. If the foci of nodular enhancement in the treated tumor is observed, a new MWA session with an identical device is performed as part of another course of treatment. When withdrawing the antenna, the needle track is coagulated with the circulated distilled water in the shaft channel, which is stopped to prevent bleeding and tumor-cell seeding.

This ablation therapy often includes a 5-10-mm ablative margin of apparently healthy tissue adjacent to the lesion to eliminate microscopic foci of disease, and the uncertainty that often exists regarding the precise location of actual tumor margin. For patients with severe liver cirrhosis or the lesion adjacent to critical organs, an ablation margin of < 5 mm or conformal ablation based on tumor shape and contours is recommended to ensure safe and radical treatment; otherwise, a 5-10-mm surgical margin is preferred. Reducing the tumor bulk or conformal ablation is the strategy for patients undergoing palliative ablation treatment.

## CARE AFTER MWA

After the MWA procedure, the punctured site is covered with a sterile dressing under pressure. The patient then undergoes recovery for 4-6 h of bed rest. The patients are observed for 2-3 additional days and discharged from the hospital when they feel no severe pain or when their body temperature does not exceed 38 °C.

## COMBINED TREATMENT WITH OTHER MODALITIES

The therapeutic efficacy of MWA can be augmented by other therapies. Similar to other thermal ablation techniques, the coagulation area of MWA is also influenced by perfusion-mediated cooling. Interruption of hepatic blood flow can significantly increase the coagulation diameters<sup>[49]</sup>. TACE is an effective method for reducing the blood flow of liver tumor because of its artery-blocking effect. When combined with MWA, it may yield increased ablation volume. MWA can destroy the remaining viable part of the tumor after TACE, whereas TACE may possibly control microscopic intrahepatic metastasis that cannot be treated by MWA<sup>[50]</sup>. As the two modalities are complementary, the combination of them is preferred, especially for treating large and multiple tumors. The combination of TACE decreases the number of microwave antenna insertions and microwave irradiation time. The decision as to whether combined therapy with TACE, intermittent treatment, or sequential therapy is adopted should be based on the patient's general condi-

tion, liver function, local tumor size and number, tumor infiltration, tumor vascularization, and reaction of tumor to local treatment. Therefore, the principle of individual treatment must be advocated.

For patients with high-risk localized tumors, combination of multiple techniques to ensure favorable effects and few complications is also recommended. Hepatic tumor in high-risk sites refers to tumor adjacent to important organs and tissues including the diaphragm, gastrointestinal tract, hilum and major bile duct or vessels. The thermal energy may spread into surrounding structures, therefore, the major concern for MWA of such tumors lies in the increased opportunity of thermal injury in the important structures. However, combined with artificial ascites, artificial pleural effusion, intraductal saline perfusion, intermittent emission of microwave antennae, and temperature monitoring assisted with small-dose percutaneous ethanol injection<sup>[51-55]</sup>, MWA becomes feasible for the treatment of dangerous site tumors without sacrificing the therapeutic efficacy.

Although US guidance has the benefits of real-time visualization of applicator placement, portability of the technology, nearly universal availability and low cost, it has several limitations including occasional poor lesion visualization as a result of a lack of innate tissue conspicuity or overlying bone- or gas-containing structures. MWA assisted by a real-time virtual navigation system is a feasible and efficient treatment of patients with lesions undetectable by conventional US<sup>[56]</sup>. Recently, 3D US-guided MWA avoids the limitation of inaccurate needle placement and the skill requirement resulting from conventional US guidance. These new techniques provide an appealing alternative option, enabling the physician to perform consistent, accurate therapy with improved treatment effectiveness<sup>[57,58]</sup>.

## FOLLOW-UP AND THERAPEUTIC EFFICACY ASSESSMENT

The Working Group on Image-Guided Tumor Ablation proposed that postprocedural follow-up of patients to assess any treatment-emergent side effects and tumor response is conducted in the first week or, at the latest, no more than 4 wk after the last course of a defined ablation protocol<sup>[59]</sup>. Subsequent routine follow-ups are then recommended every 3-4 mo. Evaluation of therapeutic effects, including technique effectiveness, local tumor progression, and complications, is recommended. The Working Group also recognized the need for close surveillance and early reintervention to achieve optimal primary tumor ablation success.

Frequent imaging studies may be required for individual patients to assess the therapeutic efficacy and to detect the intrahepatic recurrent lesion. To ensure continuity of the follow-up, most of the studies are recommended to be performed serially at the institution where the ablation is performed. The imaging studies should consist of a high-quality, contrast-enhanced CT/MRI or

US, adhering to standard scanning protocols to facilitate comparisons. Intravenous contrast is critical because pathological studies have shown that the best correlation of necrotic tissue is defined by the zone of non enhancement on cross-sectional studies<sup>[60-62]</sup>. If any areas of the ablated mass are devoid of enhancement on follow-up enhanced imaging performed 1 mo after MWA, technique effectiveness, namely complete response, is achieved<sup>[59]</sup>. Then routine contrast-enhanced US, CT or MRI and serum tumor markers are repeated to detect the local treatment response and intrahepatic and extrahepatic metastases at 3-mo intervals after MWA. If irregular peripheral enhancement in scattered, nodular, or eccentric pattern occurs in the original sites that were previously considered to be completely ablated during follow-up, which represents local tumor progression, further ablation should be considered as soon as possible if the patient still meets the criteria for MWA. US scanning is the routine baseline examination method for the ablation zone. During follow-up, the treated lesions slowly diminish in size, becoming undetectable by US, or appearing only as small hyperechoic areas or isoechoic areas with a hypoechoic rim, or simply as heterogeneous areas. On contrast-enhanced imaging, the ablation zone presents as a non-enhancement area. Additionally, positron emission tomography may be helpful in identifying distant extrahepatic metastatic disease, and it can be considered as a part of the postoperative evaluation if necessary.

Major complications of MWA are events that lead to substantial morbidity and disability, increase the level of care, or result in hospital admission or substantially lengthen hospital stay. Major complications includes bile duct stenosis, uncontrollable bleeding, liver abscess, colon perforation, skin burn and tumor seeding (Table 2)<sup>[4,5,13,19,34,36,42,63,64]</sup>. These can be controlled by surgical operation, interventional approach, or medical therapy. Side effects are undesired consequences of the procedure that, although occurring frequently, rarely if ever result in substantial morbidity. Side effects include pain, post ablation syndrome, and asymptomatic pleural effusions, which are usually self-limited and do not require any further treatments. Low-grade fever and general malaise are common manifestations of post ablation syndrome. Careful patient selection, the most appropriate imaging modality, and the best puncture routine may also help prevent complications.

## DISCLAIMER

The SCIU has written and approved the guidelines to promote the cost effective use of high-quality MWA therapeutic procedures. Percutaneous MWA techniques are recommended for use by clinical or imaging doctors with at least 3 years experience with interventional procedures. These generic recommendations cannot be rigidly applied to all patients in all practice settings. The guidelines and technology assessments are not intended to supplant physician judgment with respect to particular patients or special clinical situations, and not be deemed

**Table 2 Procedure-related complications for microwave ablation of hepatic malignancies**

Study	Intra-peritoneal bleeding	Bile duct injury	Colon perforation	Liver abscess	Skin burn	Tumor seeding	Symptomatic pleural effusion	Perioperative mortality
Sakaguchi <i>et al</i> <sup>[4]</sup>	0.51%	0.26%	0.00%	0.26%	0.77%	0.00%	1.28%	0.00%
Martin <i>et al</i> <sup>[5]</sup>	0.00%	0.00%	0.00%	2.00%	0.00%	0.00%	0.00%	0.00%
Zhang <i>et al</i> <sup>[13]</sup>	0.00%	1.25%	0.00%	0.00%	0.00%	0.00%	0.63%	0.00%
Shibata <i>et al</i> <sup>[19]</sup>	0.00%	2.78%	0.00%	2.78%	2.78%	0.00%	0.00%	0.00%
Kuang <i>et al</i> <sup>[38]</sup>	0.00%	0.00%	1.11%	1.11%	0.00%	0.00%	2.22%	0.00%
Liang <i>et al</i> <sup>[40]</sup>	0.09%	0.18%	0.18%	0.44%	0.26%	0.44%	1.06%	0.18%
Yin <i>et al</i> <sup>[46]</sup>	0.92%	0.92%	0.00%	0.00%	0.92%	0.00%	3.67%	0.00%
Dong <i>et al</i> <sup>[63]</sup>	0.00%	0.00%	0.00%	0.00%	0.85%	0.00%	0.00%	0.00%
Iannitti <i>et al</i> <sup>[64]</sup>	0.00%	0.00%	0.00%	0.00%	3.45%	0.00%	0.00%	0.00%

inclusive of all proper procedures or exclusive of other procedures reasonably directed towards obtaining the same results. Accordingly, SCIU considers adherence to this guideline assessment to be voluntary, with the ultimate determination regarding its application to be made by the physician in light of each patient's individual circumstances. At present, the guidelines have been put into practice in China by seven branches of the Chinese Medical Association, through holding standardized courses (3 finished), training and checking interventional physicians (> 300 physicians having obtained MWA licenses), and founding ablation demonstration bases (5 founded). MWA is undergoing rapid development and receiving keen interest in Europe and America, so access and training systems for MWA guidelines are expected to be recommend according to the situation in each country. The guidelines will be updated when data or publications might change a prior recommendation or when the panel feels clarifications are required for the oncology community.

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## Epigenetics of hepatocellular carcinoma: Role of microRNA

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### Abstract

Hepatocellular carcinoma (HCC) represents a major form of primary liver cancer in adults. MicroRNAs (miRs), small non-coding single-stranded RNAs of 19-24 nucleotides in length, negatively regulate the expression of many target genes at the post-transcriptional and/or translational levels and play a critical role in the initiation and progression of HCC. In this review we have summarized the information of aberrantly expressed miRs in HCC, their mechanism of action and relationship to cancer. The recent advances in HCC research reveal that miRs regulate expression of various oncogenes and tumor suppressor genes, thereby contributing to the modulation of diverse biological processes including proliferation, apoptosis, epithelial to mesenchymal transition and metastasis. From a clinical viewpoint, polymorphisms within miR-binding sites are associated with the risk of HCC. Polymorphisms in miR related genes have been shown to correlate with

survival or treatment outcome in patients. Furthermore, the review focuses on the potential role of miRs as novel biomarkers and their translational applications for diagnosis and therapy in HCC. With further insights into miR deregulation in HCC, it is expected that novel miR-based therapeutics will arise. Also, we orient the readers to other reviews that may provide better understanding of miR research in HCC.

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**Key words:** MicroRNA; Cancer; Hepatocellular carcinoma; Biomarker; Polymorphism

**Core tip:** This review provides the relationship between microRNA (miR) and hepatocellular carcinoma and speculates on the progress that will be achieved through ongoing research. A research effort to identify genetic polymorphisms associated with cancer is emphasized. The review highlights that miR-based therapeutics, and diagnostic and prognostic systems should be used for patients.

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### INTRODUCTION

Liver cancer is the second and sixth leading cause of cancer related-death in males and females respectively. Hepatocellular carcinoma (HCC) that accounts for most of the primary liver cancers is the fifth most frequently diagnosed cancer worldwide. Early detection of HCC is needed because the best indicator of prognosis is based on the stage of the disease. About 90% of HCC cases arise from cirrhosis and the disease is strongly associated with several risks factors, including hepatitis B and C

infections, alcohol abuse, primary biliary cirrhosis, autoimmune hepatitis and nonalcoholic steatohepatitis<sup>[1]</sup>. Epigenetic changes in microRNAs (miRs) and their target gene expression may provide tools and opportunities for detection and therapeutic intervention in HCC.

MiRs, a class of non-coding RNAs with lengths of 19- to 25 nucleotides (nt), act as post-transcriptional regulators by binding to 3'-untranslated region (3'UTR) of target messenger RNA (mRNA). MiRs function as endogenous suppressor of gene expression by inducing either mRNA degradation or translational repression. The promoters of *MiR* genes are regulated by transcription factors, co-activators, enhancers and suppressors similar to protein coding genes. Thus, proto-oncogene *c-myc*<sup>[2,3]</sup> and tumor suppressor *p53*<sup>[4]</sup> transactivate miRs in HCC. In a genomic cluster the individual miRs are often expressed at different times from the same pri-miR. Pri-miRs are transcribed in the nucleus into a 70-100 nt hairpin-shaped structure and the process is catalyzed by Droscha, which is associated with cofactor DGCR8 and other proteins. After translocation to the cytoplasm by Exportin5, miRs are cleaved into a 19-25 nt miR duplex by enzyme Dicer. One strand of the duplex is then incorporated into the RNA-induced silencing complex (RISC) for its mRNA targets. MiRs function as endogenous suppressor of gene expression by binding of RISC to the 3'UTR of target mRNAs and inducing either mRNA degradation or translational repression. The mRNA degradation is induced if miR binds completely or almost completely, however, if the binding is incomplete, miR represses translation of mRNA. Each step of the process is well regulated, and dysfunction at any level can result in inappropriate miR functions. Gene silencing is the most methodically studied role of miRs, however, they can up-regulate gene transcription during cell cycle arrest and, therefore, overexpression of miRs in human cancers hinted to probable oncogenic functions of miRs. As discussed earlier a direct binding of miR to 5'UTR or promoter of the target genes activate rather inhibit gene expression<sup>[5]</sup>.

Analogous to the protein-coding genes, epigenetic mechanisms, for example, CpG island hypermethylation<sup>[6-8]</sup> and histone modifications<sup>[9]</sup> also regulate miR expression in HCC. MiRs that are transcribed from CpG islands undergo DNA hypermethylation-coupled repression due to binding of the transcriptional repressor methyl CpG binding proteins. Epigenetic regulation of miRs might be more common than reported so far as approximately 16% of the annotated human miRs are located within 1000 bp of a CpG island. To date, more than 1000 human miRs have been identified and each miR control hundreds of genes. It has been suggested that miRs regulate the translation rate of more than 60% of protein coding genes.

## ABERRANT EXPRESSION OF MICRORNAS IN HCC

MiRs play a central role in basic biological processes such

as cellular differentiation, proliferation, apoptosis, migration and invasion. MiR expression profiles are different between normal tissue and derived tumors and between distinct tumor types. Protein coding genes of cell cycle, apoptosis, and metastasis are direct targets of miRs in HCC<sup>[10]</sup>. Microarray studies have identified a number of miRs that are either up-or down-regulated<sup>[11]</sup>. Down-regulation of subsets of miRs is a common finding in HCC, indicating that some of these miRs may act as putative tumor suppressor genes. Restoration of tumor suppressive miRs leads to cell cycle block, increased apoptosis and reduced tumor angiogenesis and metastasis by inhibiting migration and invasion. On the contrary, onco-miRs that are up-regulated in HCC potentially target many tumor suppressive genes. Experimental suppression of onco-miRs helps restoring expression of tumor suppressive genes that initiates apoptosis and inhibits cell proliferation, angiogenesis and metastasis in HCC. In general, to investigate the role of deregulated miRs in HCC, miR expression vectors and mature miR mimics or inhibitors (antagomirs) are transfected in HCC cell lines. Further, to confirm the target genes of respective miRs, 3'UTR luciferase vectors (empty luciferase vector or luciferase vector containing wild-type or mutant-type target gene 3' UTR) are utilized for reporter assays. Major down- and up-regulated miRs and their target genes in HCC are discussed in Table 1.

## CLINICAL SIGNIFICANCE AND TRANSLATIONAL APPLICATIONS OF MICRORNAS IN HCC

### *Single nucleotide polymorphism in miRs*

Single nucleotide polymorphisms (SNPs) in miRs and their targets have been associated with risk of various cancers. Due to the stringent recognition requirement needed by the miR and the binding region on its target gene, it is rather conceivable that SNPs could have functional implications on the post-transcriptional regulation of target genes. An SNP could either weaken a known miR target or create a sequence match to the miR that was not previously associated with the given mRNA. Changes in the expression pattern of a gene could therefore influence a person's risk of disease. Polymorphisms in miR-34b-c/rs4938723<sup>[12]</sup>, miR-101-1/rs7536540<sup>[13]</sup>, miR-101-2/rs-12375841<sup>[13]</sup>, miR-106b-25/rs99985<sup>[14]</sup> and miR-196a-2/rs11614913<sup>[15]</sup> are positively associated with HCC. On the contrary, miR-371-373/rs3859501<sup>[16]</sup> and miR-149c/rs2292832<sup>[17]</sup> are negatively associated with HCC risk. Also, a positive association of HCC risk has been demonstrated with polymorphisms in miR target genes IL-1/rs3783553 (miR-122 and miR-378)<sup>[18]</sup>, -TrCP/rs16405 (miR-920)<sup>[19]</sup>, IFNAR1/rs17875871 (miR-1231)<sup>[20]</sup>, ErbB4/rs6147150 (miR-let-7c)<sup>[21]</sup> and COL1A2/rs3917 (miR-let-7g)<sup>[22]</sup>.

### *miRs as biomarkers in HCC*

miRs are prognostic markers of HCC. Down-regulation

**Table 1** Down-regulated microRNA in hepatocellular carcinoma and their characteristics

miRs	Targets	Characteristics
Down-regulated		
miR-1	ET1	Proliferation <sup>[52]</sup>
miRs-7a, -7b, -7c, -7d, -7f-1, -7d	Caspase-3, HMGA2, C-myc, Bcl-xl	Proliferation, apoptosis <sup>[2,23,53-58]</sup>
miR-101	Mcl-1, SOX-9, EZH2, EED, DNMT3A	Proliferation, apoptosis <sup>[59-61]</sup>
miRs-122	Bcl-w, ADAM-1, Wnt-1	Angiogenesis, apoptosis, Metastasis <sup>[45,62-64]</sup>
miR-125a, -125b	MMP11, SIRT7, VEGF-A, LIN28B2, Bcl-2, Mcl-1, Bcl-w	Angiogenesis, apoptosis, metastasis, proliferation <sup>[65-70]</sup>
miR-139	c-Fos, Rho-kinase-2	Metastasis <sup>[30,71]</sup>
miR-145	IRS1-2, OCT4	Insulin-like growth factor pathway, Stem-like cells tumorigenicity <sup>[31,72]</sup>
miR-195	CDK6, E2F3, cyclinD1	Proliferation, apoptosis, tumorigenicity <sup>[73,74]</sup>
miR-199a-3p, -199-5p	c-Met, mTOR, PAK4, DDR1, caveolin-2	Proliferation, autophagy, metastasis <sup>[9,75-78]</sup>
miRs-214	HDGF, catenin	Proliferation, angiogenesis, metastasis <sup>[79-81]</sup>
Up-regulated		
miR-10a	EphA4, CADM1	EMT, metastasis <sup>[33,82]</sup>
miR-21	Pten, RhoB, PDCD4	Drug Resistance, metastasis <sup>[49,83-85]</sup>
miR-221	Bmf, DDIT4, Arnt, CDKN1B/p27, CDKN1C/p57	Angiogenesis, apoptosis, proliferation <sup>[86-89]</sup>
miRs-224	Yin Yang1/Raf-1 kinase, NFκB pathways, apoptosis inhibitor-5	Proliferation, apoptosis, metastasis <sup>[90-93]</sup>

miRs: MicroRNAs; EMT: Epithelial-mesenchymal transition.

of miR-let-7g<sup>[23]</sup>, -22<sup>[24]</sup>, -26<sup>[25]</sup>, -29<sup>[26]</sup>, -99a<sup>[27]</sup>, -122<sup>[28]</sup>, -124<sup>[29]</sup>, -139<sup>[30]</sup>, -145<sup>[31]</sup> and -199b<sup>[32]</sup> is associated with poor prognosis, increased risk of aggressive tumor recurrence and shorter disease free survival. Similarly, up-regulation of HCC associated miRs-10b<sup>[33]</sup>, -17-5p<sup>[34]</sup>, -21<sup>[35]</sup>, -135a<sup>[36]</sup>, -155<sup>[37]</sup>, -182<sup>[38]</sup>, -221<sup>[35]</sup>, and -222<sup>[35,39]</sup> is linked to poor prognosis. Studies have shown that miRs are protected from enzymatic cleavage by RNase in blood and therefore miR expression profile in serum or plasma could also be utilized as novel diagnostic markers. More than 20 miRs in serum and/or plasma have been associated with HCC detection. The expression profile of miRs-500, -92a, -25, -375 and let-7f could identify HCC cases from controls<sup>[40-43]</sup>. Furthermore, Zhou *et al*<sup>[44]</sup> demonstrated that miR-122, -192, -21, -223, -26a, -27a and -801a helped detecting early-stage HCC with high diagnostic accuracy.

### miRs as therapeutic targets in HCC

Tumor suppressive miRs that are expressed in normal liver, however, are down-regulated in tumor tissues during tumorigenesis and metastasis. For treatment, a good strategy would be to replenish such miRs systemically in HCC patients. Such miR replacement therapies have been demonstrated in the case of miRs -26a<sup>[3]</sup>, -122<sup>[45]</sup>, and -124<sup>[46]</sup> in mice models of HCC. Conversely, suppression of oncomir-221<sup>[47,48]</sup> by antagomirs resulted in prolonged survival and reduction of tumors. As targeted gene therapies are gaining interest in cancer treatment, miR as a therapeutic target would be more efficient since single miR can control multiple deranged genes in HCC. However, this hypothesis is yet to be tested in HCC patients. Further, chemotherapeutics kill normal cells and pose significant toxicities in cancer patients, a non-discriminatory behavior of chemotherapy drugs. In mice models, as discussed above, no such toxicity was observed when miRs were used to treat HCC. In this regard, miRs also influ-

ence sensitivity of tumors to anticancer drugs. Tumors with high expression of oncomir-21<sup>[49]</sup> and -181b<sup>[50]</sup> were resistant to IFN-5FU combination therapy and doxorubicin treatment respectively. A strategy to suppress these miRs by antagomirs might be useful in increasing drug efficacy. Similarly, studies have demonstrated that restoration of tumor suppressive miR-122<sup>[51]</sup> makes HCC cells more sensitive to Sorafenib treatment *via* down-regulation of multidrug resistance genes.

## CONCLUSION

miRs control expression of many target genes in HCC. miR profiling reveals molecular mechanisms of pathogenesis and hidden visions into early detection and treatment of HCC. Hundreds of miRs have been identified to date; however, computer models suggest there may be hundreds more. There are several online tools available for researchers and clinicians to identify and predict the targets of miRs. As research continues to verify *in silico* predictions, miR profiling will be a prominent tool for identification of differentially expressed miRs in HCC. More information from genome-wide association studies, assisted by high resolution SNP arrays and the next-generation sequencing technology, is anticipated to identify increasing number of polymorphisms in HCC specific miRs and their 3'UTR targets. For future studies, we should consider miRs and their regulatory networks in order to comprehend the complex processes underlying HCC transformation.

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## HBV endemicity in Mexico is associated with HBV genotypes H and G

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### Abstract

Hepatitis B virus (HBV) genotypes have distinct genetic and geographic diversity and may be associated with specific clinical characteristics, progression, severity of disease and antiviral response. Herein, we provide an updated overview of the endemicity of HBV genotypes H and G in Mexico. HBV genotype H is predominant among the Mexican population, but not in Central America. Its geographic distribution is related to a typical endemicity among the Mexicans which is characterized by a low hepatitis B surface antigen seroprevalence, apparently due to a rapid resolution of the infection, low viral loads and a high prevalence of occult B infection. During chronic infections, genotype H is detected in mixtures with other HBV genotypes and associated with other co-morbidities, such as obesity, alcoholism and co-infection with hepatitis C virus or human immunodeficiency virus. Hepatocellular carcinoma prevalence is low. Thus, antiviral therapy

may differ significantly from the standard guidelines established worldwide. The high prevalence of HBV genotype G in the Americas, especially among the Mexican population, raises new questions regarding its geographic origin that will require further investigation.

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**Key words:** Hepatitis B virus genotypes; Hepatitis B virus genotype H; Hepatitis B virus genotype G; Molecular epidemiology; Mexico; Antiviral therapy; Severity of liver disease; Clinical outcome

**Core tip:** Molecular, clinical, geographical and ethnicity evidence are characteristics that define any hepatitis B virus (HBV) genotype. All of these features are there for HBV genotype H, which is most predominant in Mexico, but not in Central America. Likewise, HBV genotype G has unique molecular characteristics and a similar route of transmission among those infected with this viral genotype, but it lacks a geographic origin. To date, despite the high prevalence of HBV genotype G cases from the Americas, especially among Mexicans, the limited number of complete sequences hinders further investigation to establish a hypothesis of an Amerindian origin.

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### INTRODUCTION

#### **Definition of hepatitis B virus genotypes and their association with human liver disease**

Hepatitis B virus (HBV) and humans share a close re-

relationship through the process of evolution and migration<sup>[1,2]</sup>. Numerous studies have demonstrated that most HBV genotypes are associated with a host population and geographical area of the world, while others tend to have a worldwide distribution, or still remain unknown<sup>[3,4]</sup>.

In 1988, Okamoto *et al.*<sup>[3]</sup> proposed the first genetic classification for HBV strains, defining a genotype as a viral sequence with an intertypic nucleotide divergence of more than 8% based on the entire genome. Later, a 4.2% nucleotide divergence using the S gene sequence was proposed by Norder *et al.*<sup>[5]</sup>. Throughout their discovery, each new genotype was defined by the same criterion and designated with letters in an alphabetical order, from A to J. However, given the wide diversity of HBV genomes worldwide, several authors have proposed over the years that precise criteria be fulfilled in order to identify and describe a specific genotype/subgenotype<sup>[6-9]</sup>. Recently, Kurbanov *et al.*<sup>[10]</sup> have endorsed and updated these recommendations which, in summary, are the following: use of whole genome sequences, divergence of  $\geq 7.5\%$  ( $> 4\%$  to  $< 7.5\%$  in the case of a subgenotype), strong independent clustering on molecular evolutionary analysis, avoidance of recombinants, as well as substantial epidemiological, virological and clinical evidence.

Regarding these latter points, in 2002, Chu *et al.*<sup>[11]</sup> raised key questions about the association of HBV genotypes with clinical practice: (1) “What is the predominant HBV genotype in each country or geographic region?” (2) “Is the geographic distribution of HBV genotypes related to the endemicity of HBV infection?” (3) “Is there a correlation between HBV genotype and HBV replication activity of liver disease, clinical outcome and treatment response?” and (4) “Is there a correlation between HBV genotype and risk of progression to chronic infection?”

Accordingly, the geographic distribution of HBV genotypes in regard to their regional host population and endemicity has been widely considered. In general, HBV genotypes B and C are associated with the populations of the Asian countries<sup>[3]</sup> while genotypes A and D are prevalent among European countries and the United States<sup>[3]</sup>; genotypes E and F are confined to countries of the African continent<sup>[12]</sup> and the Americas<sup>[12]</sup> (Central and South America), respectively. HBV genotype G (HBV/G) was originally reported in France<sup>[13]</sup> but has a global distribution, and HBV genotype H (HBV/H) was first revealed in Central America<sup>[14]</sup>. HBV genotypes I and J have been reported in dispersed regions of Asia and Japan, respectively<sup>[15,16]</sup>. Likewise, the genetic diversity, disease progression and response to antiviral therapy<sup>[17-20]</sup> of the European and Asian genotypes (A-D)<sup>[21-23]</sup> have received greater attention than those that are typically prevalent in the western hemisphere (E-H)<sup>[24-27]</sup>, while evidence about genotypes I and J is insufficient to respond to such arguments<sup>[10,28]</sup>.

### **Milestones in the discovery of HBV genotypes G and H worldwide and in Mexico**

HBV/G and HBV/H were revealed almost at the same time. Both discoveries represent the culminating results

of investigations carried out in the 1990s by many different laboratories worldwide. HBV/G was first described as an HBV variant<sup>[29,30]</sup> and formally reported by Stuyver *et al.*<sup>[13]</sup> in 2000. In our laboratory, HBV/G was detected in 2000, but not reported until 2002 by Sánchez *et al.*<sup>[31]</sup>. Further on, research studies focused on the development of molecular diagnostic methods<sup>[32]</sup> and the relationship between clinical and virological characteristics in comparison with the other known genotypes<sup>[26,33]</sup>. However, unlike the rest of them, the geographic origin of HBV/G is still unknown<sup>[34]</sup>.

As for HBV/H, Dr. Norder from Sweden and two other laboratories, Dr. Misokami from Japan and Dr. Panduro in Mexico, were studying the genetic variability of HBV that resulted in the identification of HBV/H in the last decade of the preceding century. However, the first HBV/H strains from Mexico were classified as HVB genotype F (HBV/F) by Sánchez *et al.*<sup>[31]</sup> since complete sequences of genotype H were not available for comparison. Later, after discussing our findings with Dr. Norder in Mexico, two strains from Nicaragua and one from the United States were made known as the new genotype H<sup>[14]</sup>. Since then, HBV/H has often been referred to as from Central America, because of the two original Nicaraguan strains. Further discussion regarding the validity of genotype H was provided by Kato *et al.*<sup>[35]</sup>, given that seven HBV isolates (doubtfully H) differed from a number of selected HBV/F strains by a genetic divergence of 7.3%-9.5%, thus proposing a new subtype (F2) of HBV/F.

Overall, in the last ten years, the knowledge on HBV/H regarding the relationship between virological-clinical characteristics and its geographical and host population prevalence has increased significantly, allowing us to have a better understanding of HBV-infected patients. Herein, we provide an updated overview of such evidence concerning the endemicity of HBV infection based on genotypes H and G in Mexico.

## **HBV GENOTYPE H**

### **Molecular characteristics of HBV genotype H**

In the study by Arauz-Ruiz *et al.*<sup>[14]</sup>, the three original samples (1853Nic, 2928Nic, LAS2523) designated as HBV/H diverged from selected genotype F strains by 7.2%-10.2%. In the polymerase region, the three strains had 16 unique conserved amino acid residues not present in genotype F strains. Additionally, HBV/H also differs from them by two distinct substitutions in the surface antigen protein, at Val<sup>44</sup> and Pro<sup>45</sup>, as well as at Ile<sup>57</sup>, Thr<sup>140</sup>, Phe<sup>158</sup> and Ala<sup>224</sup><sup>[4]</sup>. Furthermore, by “TreeOrder Scan” analysis, genotype H strains show evidence of recombination with genotype F within the small S gene (nucleotide 350-500)<sup>[1]</sup>.

As mentioned before, the limited number of sequences available at that time made it difficult to distinguish HBV/H as an independent genotype, due to its close phylogenetic relatedness with HBV/F<sup>[14,35]</sup>. Nevertheless, the amount of HBV/H sequences reported in GenBank

has increased; hence pair-wise analysis of complete sequences of HBV/F compared against the latest Mexican HBV/H strains result in a genetic distance of at least 0.08 (data not shown). Thus, the initial differences reported by the authors could have been related to the fact that the earlier isolates came from subjects with residence outside of Mexico<sup>[14,35]</sup>.

Additionally, the estimated maximum likelihood phylogeny of HBV/H and HBV/F genomes exhibits a distinct genetic divergence from a common ancestor while HBV/H sequences tend to cluster into multiple and nested clades<sup>[36]</sup>. Further phylogeographic studies based on coalescent models are necessary to provide fresh information regarding these evolutionary characteristics, and integrate them to the timeline of migrations of the prehispanic people from ancient Mexico towards South America.

### **Molecular epidemiology of HBV genotype H in Mexico**

During the last 10 years, the geographic origin of HBV/H was referred to as from Central America. Today, it is clearly evident that most HBV/H sequences deposited in GenBank are from Mexico, while those isolated worldwide come from individuals reporting sexual relationships with people from the Americas, and no further H strains have been reported from Central America<sup>[36]</sup>.

Epidemiological studies using only hepatitis B surface antigen (HBsAg) determinations have shown a steady prevalence rate of 0.3% since 1976 to date, ranking Mexico as a region of low endemicity<sup>[37]</sup>. However, by anti-HBc marker and molecular diagnosis of HBV genomes, high endemic areas of HBV infection have been detected in the native population<sup>[37-39]</sup>, similar to the indigenous populations of the Central and South American countries<sup>[40]</sup>.

It has been estimated that nearly 15 million Mexican adults have been infected by HBV during their lifetime, since the anti-HBc prevalence increases with age<sup>[41,42]</sup>. Additionally, estimates suggest that at least another 5 million native people could be at risk of acquiring infection<sup>[41]</sup>. HBV/H infection is acquired primarily during adulthood by horizontal transmission, through sexual relationships and contact with contaminated body fluids, which could explain why the majority of infected patients do not develop chronic liver disease<sup>[41]</sup>.

HBV/H is the predominant genotype in asymptomatic infected patients living in high endemic areas<sup>[36,38]</sup>, as well as in patients with acute and chronic liver disease<sup>[43,44]</sup>. Indeed, HBV/H is prevalent in more than 90% of the cases, followed by HBV/A, HBV/D and HBV/G, whereas other known HBV genotypes are rare<sup>[36]</sup>. Furthermore, the predominance of HBV/H in Mexico is historically related to the migrations of the prehispanic people, the settlement of the Aztecs in Mesoamerica before the Spanish conquest and the successive admixture of the population; hence, it is the predominant genotype detected in both Mexican native (Amerindian) and mestizo populations<sup>[36,38]</sup>.

### **Clinical presentation of HBV/H infected patients**

HBV/H infected patients usually are asymptomatic without clinical or laboratory manifestations of liver disease<sup>[38,43]</sup>; thus, the existence of liver damage is modest or undetectable, whereas occult B infection (OBI) is a common manifestation<sup>[38,45]</sup>. This situation may be attributed to a rapid resolution of the disease, associated with the genetic characteristics of either the virus or the host<sup>[46]</sup>.

HBV/H is often detected in patients with acute liver damage. This clinical symptom is observed in male patients, such as men who have sex with men, mainly during the acute phase, and then with viral clearance or OBI after acute infection and flares during immunosuppressive conditions<sup>[46]</sup>. In chronic patients, HBV/H is predominant also; however, OBI is common so that the association of the HBV/H with the progression and severity of liver disease is masked by the presence of other co-morbidities, such as alcoholism, obesity and co-infection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV)<sup>[36]</sup>. HBV/H-infected patients tend to have low viral loads, usually < 4000 UI/L, that are easily detected as they increase, up to 100000 UI/L or above when patients are infected with mixtures of HBV genotypes<sup>[36]</sup>.

Lastly, the low prevalence of HBV infection among the Mexican population is associated with a low prevalence of hepatocellular carcinoma (HCC) from 1953 to date<sup>[47-49]</sup>. This is contrary to what occurs in the Asian countries, where acute and chronic HBV infection with genotypes B and C and HCC are highly prevalent. Thus, the predominance of HBV/H among the Mexican population is associated with low endemicity, low viral load, minimum cases of acute and chronic liver diseases due to HBV infection and a low prevalence of HCC.

This matter raises a word of caution regarding the strategy for antiviral therapy in Mexico since the main cause of liver disease may be attributed to co-morbidities, such as HCV or HIV infection, alcoholism or obesity, but not to HBV infection exclusively. Therefore, antiviral therapy for HBV/H-infected patients should be given with precaution until the usefulness of the conventional antivirals is fully demonstrated for this genotype, considering that the international guidelines for the treatment of chronic B infection have been designed for populations of other geographic regions that have different HBV genotypes, endemicity and progression of liver disease.

## **HBV GENOTYPE G**

### **Molecular characteristics of HBV genotype G**

The HBV/G genome has some unique characteristics. It contains a 36-nt/12 amino acid insertion with pleiotropic effects on core protein expression, genome replication and virion secretion, not found in any other HBV genotype<sup>[50]</sup>. It also has two stop codons in the preC region at position 2 and 28, which prohibits the translation of the hepatitis B e antigen (HBeAg)-precursor; thus, patients

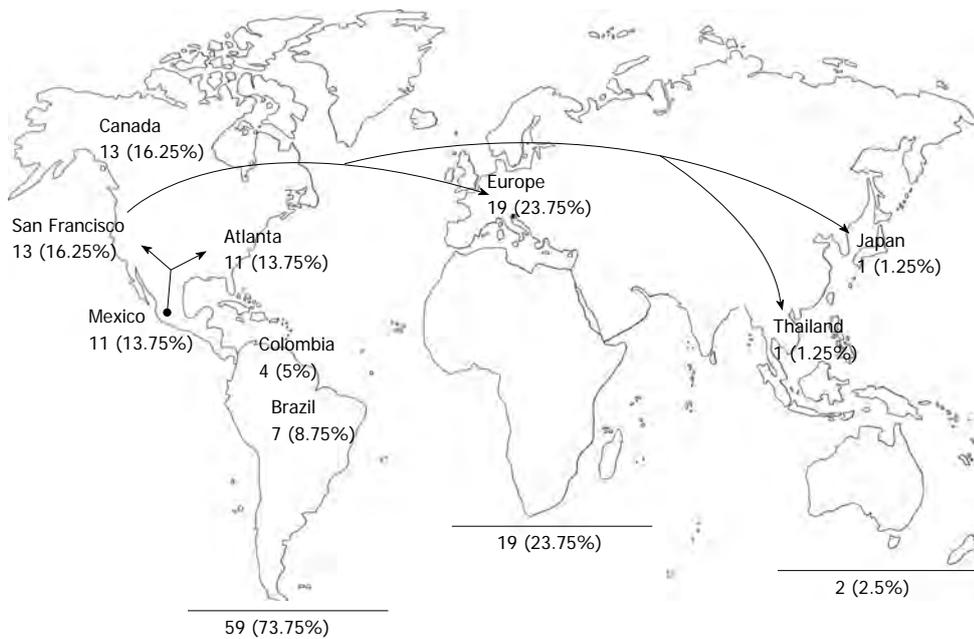


Figure 1 Geographic distribution of the worldwide epidemiology of the hepatitis B virus genotype G isolates. Fifty-nine genome sequences out of a total of 80 have been reported from the Americas (73.75%), whereas 23.75% are from Europe and 2.5% from Asia. The high prevalence of hepatitis B virus genotype G in America may be related to a common source of infection and transmission route.

who are mono-infected with HBV/G are negative for HBeAg<sup>[51]</sup>. Other molecular characteristics include two deletions, one at the carboxyl terminal region of HBcAg and another in the preS1 region<sup>[51]</sup>.

At the nucleotide level, the majority of the complete genome sequences of HBV/G strains share a remarkable sequence conservation of more than 99%<sup>[52]</sup>. Furthermore, there is a high nucleotide similarity within the S gene sequence (94.6%-97.5%), considered as evidence of recombination with genotype A (HBV/A) in the small S fragment (nucleotide 250-350)<sup>[11]</sup> and a 30 base pair fragment in the preS region that is almost identical to genotype E<sup>[34]</sup>.

### Molecular epidemiology of HBV/G in Mexico

Worldwide, a significant number of HBV/G strains have been detected in men who have sex with men<sup>[13,26,29,30,52-54]</sup>, suggesting that sexual genital-anal contact may play a significant role in the transmission of HBV infection<sup>[55]</sup>. However, parenteral transmission has been reported, mainly as mono-infection, such as in blood donors<sup>[56-58]</sup> and hemodialysis patients<sup>[59]</sup>.

In the past years, several publications continue to report that little is known about the geographical origin of HBV/G, and yet it is considered ubiquitous. Such statements have arisen due to earlier HBV/G cases reported from France<sup>[13]</sup>, Germany<sup>[54]</sup> and the cities of San Francisco, CA<sup>[26,29,52]</sup> and Atlanta, Georgia<sup>[13]</sup> in the United States. However, despite the limitations of using RFLPs or strip molecular methods for the detection of HBV/G, instead of complete genome sequences<sup>[10]</sup>, most of the cases of this genotype have been reported from the Americas (73.5%), including Mexico<sup>[31,38,46,55,60,61]</sup> and to a

lesser degree from Europe<sup>[30,50,56,62-65]</sup> (23.75%) and other regions of the world (2.5%)<sup>[66-68]</sup> (Figure 1).

As mentioned before, the geographic origin of HBV/G is still unknown, due to its low global prevalence combined with the lack of epidemiological and clinical data. The genomic characteristics of this genotype are puzzling. On one hand, HBV/G complete sequences share such a close similarity that a specific molecular epidemiological route of transmission among the international cases or a simple evolutionary history cannot be elaborated. On the other hand, the similarity of certain regions of the HBV/G genome with genotypes A<sup>[1]</sup> and E<sup>[34]</sup> suggest co-evolutionary processes among themselves<sup>[10]</sup>. These features have created considerable difficulties to pinpoint a distinct geographic origin for HBV/G.

A hypothesis on a plausible African geographic origin of HBV/G was proposed by Lindh<sup>[34]</sup> in 2005, based on its similarity with HBV/E which is prevalent in Africa and that the worldwide spread of HIV infection from Africa may have been the cause of the dispersion of HBV/G. Unfortunately, HBV/G African sequences have not been deposited in GenBank nor have G/E recombinants been associated with a host population to date. Furthermore, based on the low genetic diversity of HBV/E (1.67%) and its short evolutionary history<sup>[69]</sup>, it has been suggested that it was introduced into the African population after the Atlantic slave trade<sup>[69-71]</sup>. This is consistent with the fact that HBV/E is virtually absent in the Americas, despite the significant number of African slaves introduced into the United States and Latin America (including Mexico), both regions having a high presence of black population, the former of Afro origin and the latter mixed descendents of a large black popula-

tion forced into slavery. Thus, the worldwide spread of HBV/G appears to have not co-dispersed HBV/E, since G/E recombination or G-E co-infection is absent among the admixture populations. Interestingly, the similarity of the 150 base pair fragment between HBV/A and HBV/G could be related to the most common dual HBV G/A infection reported in the United States<sup>[26,52]</sup>, Canadian<sup>[53]</sup> and European cases<sup>[56]</sup>. Given that HBV/A is common in Europe, it may be speculated that genotype G could have reached the Americas by way of the Caucasian people. However, despite the fact that HBV/A is a minor strain in Mexico<sup>[36]</sup>, HBV G/H co-infection is more frequent than G/A<sup>[55]</sup>. Thus, G/H co-infection may be related to the plausibility that genotype G is endemic to Mexico, as well as genotype H. Furthermore, HBV/G has been detected in patients with chronic liver disease; pathogenesis of liver fibrosis has been documented in *in vitro* experiments<sup>[60,72]</sup> and corroborated in patients with co-infection with other HBV genotypes.

The relationship of HBV/G sequences with the Mexican population is based on the following observations: (1) the high prevalence of HBV/G sequences in the American continent (73.75%) (Figure 1); (2) 16 HBV/G cases were detected among 77 HIV/HBV co-infected individuals (21%)<sup>[61]</sup>; (3) 5 HBV/G cases out of 49 high risk individuals (10.2%)<sup>[27]</sup>; and (4) HBV/G sequences have been identified in an ongoing study cohort of young children with HBV infection in our laboratory. These findings lead us to ask ourselves: "Is HBV/G endemic to the Americas, including Mexico, Colombia, and Brazil or was it introduced into the continent?" The evidence that could support an Amerindian hypothesis requires that sequences from native and mestizo populations be analyzed. To date, 11 sequences from Mexico<sup>[31,38,55,60]</sup>, 7 from Brazil<sup>[73-75]</sup> and 4 from Colombia<sup>[58]</sup> have been retrieved from mestizo populations, except for one Mexican case belonging to a native from the Huichol community<sup>[38]</sup>. The presence of HBV/G in this community could be explained by the fact that native individuals engage in multi-partner sexual relationships and male-to-male sexual activity<sup>[38,76,77]</sup>. However, further phylogeographic studies are required in order to determine if these findings may be related to the transmission of HBV/G infection among distinct Amerindian communities before the global dissemination of blood-borne infectious diseases.

Nevertheless, Mexican and United States HBV/G strains share a close genetic homology<sup>[55]</sup>. This is consistent with the fluent migration events that have occurred for centuries across the United States-Mexican border, especially from the western states of Jalisco, Michoacan and Guanajuato, towards the large United States Hispanic communities, such as in Los Angeles, San Francisco and Atlanta, among others<sup>[78]</sup> (Figure 1). However, despite this feasible epidemiological association, further evidence is required to verify if the transmission of HBV/G infection may have occurred among same-sex couples/transgender individuals traveling to and from Mexico and the United States<sup>[79,80]</sup>.

## CONCLUSION

The predominance of HBV genotype H among the Mexican population is associated with a definite geographic region and historical context. The endemicity of HBV infection in Mexico manifests with a low HBsAg seroprevalence, due to a rapid response to the infection, low viral loads and a high prevalence of occult B infection. During chronic infections, HBV infection may be undetectable and associated with co-morbidities, such as obesity, alcoholism and co-infection with HCV or HIV. These manifestations correlate with the low prevalence of hepatocellular carcinoma. Based on these features, antiviral therapy may differ significantly from the international guidelines that have been established for patients within the regions of high endemicity. As for the high prevalence of HBV/G cases reported in Mexico, more detailed phylogenetic analysis of other HBV/G complete sequences will be required in order to elucidate its geographic origin.

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## Biliary phosphatidylcholine and lysophosphatidylcholine profiles in sclerosing cholangitis

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### Abstract

**AIM:** To analyze phospholipid profiles in intrahepatic bile from patients with primary sclerosing cholangitis (PSC) and secondary sclerosing cholangitis (SSC).

**METHODS:** Intrahepatic bile specimens collected *via* endoscopic retrograde cholangiography from 41 patients were analyzed. Fourteen of these patients were diagnosed with PSC, 10 with SSC, 11 with choledocholithiasis or no identifiable biliary disease, and 6 with cholangiocellular carcinoma (CCC). Bile acid, cholesterol, and bilirubin contents as well as pancreas

lipase activity in bile were determined by biochemical methods. Phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) species were quantified using nano-electrospray ionization tandem mass spectrometry.

**RESULTS:** Bile from all the examined patient groups showed a remarkably similar PC and LPC species composition, with only minor statistical differences. Total biliary PC concentrations were highest in controls ( $8030 \pm 1843 \mu\text{mol/L}$ ) and lowest in patients with CCC ( $1969 \pm 981 \mu\text{mol/L}$ ) ( $P = 0.005$ , controls *vs* SSC and CCC, respectively,  $P < 0.05$ ). LPC contents in bile were overall low ( $4.2\% \pm 1.8\%$ ). Biliary LPC/PC ratios and ratios of biliary PC to bilirubin, PC to cholesterol, PC to protein, and PC to bile acids showed no intergroup differences.

**CONCLUSION:** PC and LPC profiles being similar in patients with or without sclerosing cholangitis, these phospholipids are likely not of major pathogenetic importance in this disease group.

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**Key words:** Primary sclerosing cholangitis; Secondary sclerosing cholangitis; Cholangiocellular carcinoma; Phosphatidylcholine; Lysophosphatidylcholine; Bile; Mass spectrometry

**Core tip:** Based on the idea that unfavorable alterations of biliary phospholipids might play a role in the pathogenesis of sclerosing cholangitis, phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) species profiles were analyzed in endoscopically-acquired intrahepatic bile using nano-electrospray ionization tandem mass spectrometry. The examination of specimens from 14 patients with primary sclerosing cholangitis, 10 patients with secondary sclerosing cholangitis, 11 patients with choledocholithiasis/no biliary disease and 6 patients with cholangiocellular carcinoma revealed strikingly

similar PC and LPC species patterns, implicating at the most a minor role of biliary phospholipid changes in sclerosing cholangitis.

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## INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease with fibrobliterative sclerosis of intra- and/or extrahepatic bile ducts, eventually leading to biliary cirrhosis<sup>[1,2]</sup>. The etiopathogenesis of the disease is not yet completely understood<sup>[3]</sup>.

Secondary sclerosing cholangitis (SSC) also belongs to the group of chronic sclerosing cholangitis. SSC is thought to develop as a consequence of known injuries or secondary to pathological processes of the biliary tree<sup>[4]</sup>. The mechanisms leading to cholangiopathy in critically ill patients are mostly unknown; however, the available clinical data indicate that ischemic injury to the intrahepatic biliary tree may be one of the earliest events responsible for the development of this severe form of sclerosing cholangitis. Therapeutic options for most forms of SSC are limited, and patients with SSC who do not undergo transplantation have significantly reduced survival compared to those with PSC. Sclerosing cholangitis in critically ill patients, in particular, is associated with rapid disease progression and poor outcome<sup>[4,5]</sup>. PSC and SSC can be treated successfully only by liver transplantation.

Genetic or chemical modifications of bile composition have been found to induce sclerosing cholangitis and liver fibrosis in a number of animal models, which gave rise to the “toxic bile” concept<sup>[6]</sup>. Bile contains various biochemical components whose alterations could lead to an imbalance between its protective and harmful effects, thus leading to chronic inflammation and, finally, to the destruction of small and large bile ducts. These alterations could be primary or secondary to inflammatory processes of different origins.

Phospholipids are an essential ingredient of bile. They represent one of its major lipid components besides cholesterol and bile salts. Among bile phospholipids, there are mostly mixed diacylphosphatidylcholines. They have a hydrophilic, zwitter-ionic phosphocholine head group and two hydrophobic fatty acid side chains<sup>[7]</sup>. Phospholipids are considered to potently emulsify hydrophobic molecules, such as certain bile acids, and thereby attenuate their toxicity<sup>[8]</sup>. If it can be shown that they are unfavorably altered in concentration or species composition, then

the “toxic bile” concept could be considered relevant in the pathogenesis of sclerosing cholangitis, especially the one of PSC.

To date, no reports have been published on the comparison of the biliary phospholipid composition in PSC patients, SSC patients, and controls. The present study was aimed at determining their potential differences, especially concerning the phosphatidylcholine (PC) species composition in bile, which could help obtain further insight into the pathogenesis of sclerosing cholangitis and might be useful as a diagnostic tool for easier differentiation between biliary diseases of various origins. The hypothesis that alterations in the phospholipid composition of bile are involved in the pathogenesis of sclerosing cholangitis is supported by the fact that mice with targeted disruption of the *Mdr2* (*Abcb4*) gene, which encodes canalicular phospholipid flippase, spontaneously develop cholangitis and typical onion-skin-type periductal fibrosis, which mirrors some of the key features of human PSC<sup>[9,10]</sup>. However, the composition of bile in PSC patients without elevated serum bilirubin has been shown to be normal<sup>[11]</sup>. Furthermore, the role of *MDR3* variants in the pathogenesis of PSC in humans is not yet clear<sup>[12]</sup>. Our group showed a reduced PC/bile acid ratio in bile from a patient suffering from inborn chronic cholestatic liver disease with fibrosis. He had a homozygous missense mutation of *Abcb4* encoding *MDR3*<sup>[13]</sup>. These findings still suggest that changes in biliary lipid composition and total concentrations could play an important role in the pathogenesis of PSC and maybe SSC, which seem to differ in etiological factors and pathogenesis, but have a similar phenotype, although the latter, being recently identified, has been scarcely described. Also to be mentioned in this context are previous publications dealing with potential protective effects of phospholipids, especially glycerophospholipids, in other liver diseases<sup>[14]</sup>. For example, it was shown that alcohol-induced hepatic fibrosis could be alleviated by polyunsaturated lecithin<sup>[14,15]</sup>.

In the light of the above-mentioned data and theories, we focused on a systematic electrospray mass spectrometric analysis of bile phospholipids in two types of sclerosing cholangitis and compared them to the data from specimens of patients with choledocholithiasis or malignant biliary disease. If the bile phospholipid composition was involved in the pathogenesis of sclerosing cholangitis, one would expect differences in phospholipid concentrations and/or species patterns between bile from patients with or without sclerosing cholangitis. The evaluation of the ratios of PC concentrations to bile acid as well as lysophosphatidylcholine (LPC) concentrations are particularly interesting in that respect, since certain bile acids and LPC are thought to have cytotoxic properties<sup>[16]</sup> which might be alleviated by PC.

## MATERIALS AND METHODS

Hepatic bile specimens were collected from the following four groups of patients: controls, PSC patients, SSC

**Table 1** Clinical characteristics of the four included patient groups

	Controls ( <i>n</i> = 11)	PSC ( <i>n</i> = 14)	SSC ( <i>n</i> = 10)	CCC ( <i>n</i> = 6) with PSC ( <i>n</i> = 2)	<i>P</i> value
Gender (M/F)	9/2	10/4	10/0	3/3	
Age at ERC (yr)	52.8 ± 6.6	41.1 ± 2.3	52.1 ± 4.0	64.5 ± 5.2	0.02; PSC <sup>a</sup>
Serum albumin level (g/L)	41.5 ± 1.5 (ND in 3)	39.2 ± 1.9	37.6 ± 3.1 (ND in 5)	34.5 ± 1.4	0.13
Serum AP level (U/L)	221.3 ± 99.9 (ND in 1)	271.5 ± 37.1	1004 ± 304 (ND in 3)	357 ± 62.4	0.003; controls <sup>d</sup>
Serum bilirubin level (mg/dL)	1.6 ± 0.5 (ND in 1)	2.7 ± 0.8	5.3 ± 2.3 (ND in 2)	4.9 ± 1.9	0.39
Sterile bile or scarce bacterial growth/moderate or abundant bacterial growth	6/5	9/5	5/5	4/1 (ND in 1)	
Intake of UDCA (yes/no)	0/11	13/1	6/2 (2 unknown)	3/3	
Dominant bile duct stenosis (yes/no)	0/11	4/10	1/9	6/0	
Cholelithiasis and/or sludge (yes/no)	9/2	0/14	2/8	0/6	
Diagnosis of inflammatory bowel disease (yes/no)	0/11	10/4	0/10	2/4	

M: Male; F: Female; PSC: Primary sclerosing cholangitis; AP: Alkaline phosphatase; ERC: Endoscopic retrograde cholangiography; UDCA: Ursodeoxycholic acid; ND: Not done. <sup>a</sup>*P* < 0.05 vs Cholangiocellular carcinoma (CCC); <sup>d</sup>*P* < 0.01 vs Secondary sclerosing cholangitis (SSC).

patients, and patients with cholangiocellular carcinoma (CCC). Clinical data originated from a data base predominantly set up for the collection of samples from PSC patients. As the sample collection is also used for other studies, only specimens with sufficient amounts of material left could be used. A maximum number of 14 patients per group was predefined. Fourteen PSC patients were randomly chosen from the sample bank by a technical assistant who was not involved in phospholipid measurements. For the other groups, less than 14 samples in every group were available. Clinical data were extracted from the database, which had been set up prospectively for a wide array of research projects. The clinical characteristics of the included patient groups are presented separately in Table 1. All procedures in this study were compliant with the Declaration of Helsinki and approved by the local ethics committee. All patients had provided written informed consent before their specimens and data were included in the database. Bile samples were collected during endoscopic retrograde cholangiography (ERC) at the Department of Endoscopy at the University Hospital Heidelberg between 2007 and 2012. For all groups, the serum albumin level, as a parameter of liver synthesis function, was within the normal range.

### Control group

The control group (11 patients in all; two women and nine men; aged 24 to 81 years) comprised nine patients with cholelithiasis without signs of relevant cholangitis when ERC was performed. The remaining two patients had undergone ERC for unexplained elevation of serum alkaline phosphatase (AP) levels; one of them had recurrent right side abdominal pain. ERC findings in both of these patients were completely normal.

### Patients with PSC

PSC was diagnosed on the basis of typical ERC findings. Of the 14 patients with PSC (four women, ten men; aged

25 to 55 years), four had no inflammatory bowel disease, nine had ulcerative colitis, and one suffered from Crohn's disease. The mean disease duration of PSC at sample acquisition was 8.9 ± 1.4 years. Four of the patients underwent endoscopic dilation for dominant stenosis of a major bile duct during the ERC. All the PSC patients received ursodeoxycholic acid (UDCA) at doses between 1000 and 1500 mg per day, except one patient who had to discontinue the drug due to adverse effects. More detailed information is provided in Table 1.

### Patients with SSC

Patients who had cholestatic liver disease with the ERC morphology of SSC without evidence of pre-existing hepatobiliary disease and who had previously required long-term treatment in an intensive care unit were included. Polytrauma and sepsis were the main reasons for long-term intensive care treatment among the patients. Ten patients (all male; aged 35 to 70 years) were included. The mean disease duration since first diagnosis of SSC was less than one year. None of these patients suffered from inflammatory bowel disease. Two of the patients did not take UDCA; six were on UDCA at daily doses between 500 and 1500 mg, and for two patients, the history of medication was unknown. Further clinical data are indicated in Table 1.

### Patients with CCC

All patients in this group were diagnosed with CCC on the basis of morphological and histological findings. They all had relevant stenosis of the common bile duct and had visited the hospital for a change in or the insertion of a bile duct endoprosthesis. Of the eight patients who were initially to be included, two had to be excluded since their bile was colorless and phospholipids were below the limit of detection. This could be explained by massive cholestasis in these two patients. Two of the six patients who were finally included had PSC-related CCC,

**Table 2** Phospholipids used as internal standards to determine phospholipid concentrations in bile

Fatty acid(s)		Molecular structure name	Provider
LPC standards			
482 Da	15:00	1-pentadecanoyl-2-hydroxy-sn-glycero-3-PC	Avanti (Alabaster, AL, United States)
552 Da	20:00	1-arachidoyl-2-hydroxy-sn-glycero-3-PC	Avanti (Alabaster, AL, United States)
PC standards			
622 Da	12:0/12:0	1, 2-didodecanoyl-sn-glycero-3-PC	Sigma (Deisenhofen, Germany)
678 Da	14:0/14:0	1, 2-tetradecanoyl-sn-glycero-3-PC	Sigma (Deisenhofen, Germany)
846 Da	20:0/20:0	1, 2-dieicosanoyl-sn-glycero-3-PC	Sigma (Deisenhofen, Germany)
902 Da	22:0/22:0	1, 2-didocosanoyl-sn-glycero-3-PC	Sigma (Deisenhofen, Germany)

PC: Phosphatidylcholine.

and four had CCC unrelated to PSC. All the patients with PSC-related CCC and one of the other patients were on UDCA at daily doses between 750 and 1500 mg (Table 1).

### Collection and storage of bile specimens

For endoscopic collection of bile specimens, the papilla of Vater was selectively cannulated. Bile samples were obtained by suction and, if possible, before injection of contrast medium and any therapeutic procedure. In patients with whom bile collection was not possible before injection of contrast medium into the bile duct, a volume equivalent to that of the contrast medium was first extracted by suction into a syringe to be discarded before the syringe for the actual bile specimen was attached. This was performed in order to minimize effects of dilution. All specimens were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  before further use. Bile specimens from 41 patients were included in this study (see above).

### Determination of levels of pancreas lipase activity, protein, cholesterol, bile acids, and bilirubin in bile

As an indicator of the amount of refluxing pancreatic juice in the bile specimens, pancreas lipase activity was determined photometrically by using the chromogenic lipase substrate DGGMR (1,2-O-dilauryl-rac-glycero-glutaric acid ester)<sup>[17]</sup>. Total protein concentrations in the bile were determined using the 2-D-Quant kit (Amersham Biosciences, Amersham, United Kingdom). Bile cholesterol levels were determined by the CHOD-PAP enzymatic photometric test ("Cholesterol FS\*", Diagnostic Systems International GmbH, Holzheim, Germany)<sup>[18]</sup>. Total bile salt concentrations were measured spectrophotometrically by using  $3\alpha$ -hydroxysteroid dehydrogenase<sup>[19]</sup>. Biliary bilirubin concentration was determined using the Jendrassik-Grof method<sup>[20]</sup>.

### Bacterial cultures

Aliquots of fresh bile specimens were sent to our Department of Microbiology directly after acquisition for

aerobic and anaerobic bacterial cultures. Bacterial growth was semiquantitatively described as non-existent, scarce, moderate, or abundant.

### Lipid extraction from bile specimens

Extraction of lipids from bile specimens was performed according to Folch<sup>[21]</sup>. One-microliter aliquots of bile were diluted in 75  $\mu\text{L}$  distilled water each. Four non-physiological PC and two non-physiological LPC standards were added before extraction. Both these phospholipid classes constitute more than 95% of biliary phospholipids<sup>[7]</sup>, and no additional phospholipid standards were used. Crude lipid extracts were completely dried. For mass spectrometry, each sample was redissolved in 50  $\mu\text{L}$  of methanol/chloroform 2:1 (v/v). Table 2 provides detailed information about the phospholipid standards used. It includes molecular weights (Da), numbers of carbon atoms and double bonds of fatty acids, names of the molecules as well as their structures and the companies where they were purchased.

### Nano-electrospray ionization tandem mass spectrometry

Mass spectrometry (MS) analyses were performed using a triple quadrupole instrument (Finnegan MAT, San Jose, CA, model TSQ 7000) with a nano-electrospray source operating at a typical flow rate of 20-50 nL/min. The electrospray capillary was positioned at a distance of 0.5-1 mm from the orifice of the heated transfer capillary ( $140^{\circ}\text{C}$ ). Argon was used as the collision gas (2 mTorr). Lipid extracts were infused into the heated capillary. The mass spectrometric resolution was set to the approximate nominal mass resolution for the scan range of an m/z of 400-1000. All specimens were analyzed in the precursor ion-scan mode for an m/z of 184. At least 100 consecutive scans of four seconds each were averaged for every measurement. After comparison of all spectra, the most abundant physiological PC and LPC species were identified. For the quantification of physiological phospholipid species, regression curves were determined from the non-physiological standards as per the method described by Brügger *et al.*<sup>[22]</sup>. A typical spectrum from a PSC patient is presented in Figure 1. Total amounts of PC and LPC were calculated by addition of all single species.

### Statistical analysis

The results have been expressed in terms of mean and standard error of the mean (SE) values. Due to small sample sizes, no assumptions of normality were made, and non-parametric tests (Kruskal-Wallis test and Dunn's post-test) were used to compare disease groups. *P* values of  $< 0.05$  were considered to be statistically significant. All statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Inc., CA).

## RESULTS

### Clinical data

Statistical differences in the clinical data of the four pa-

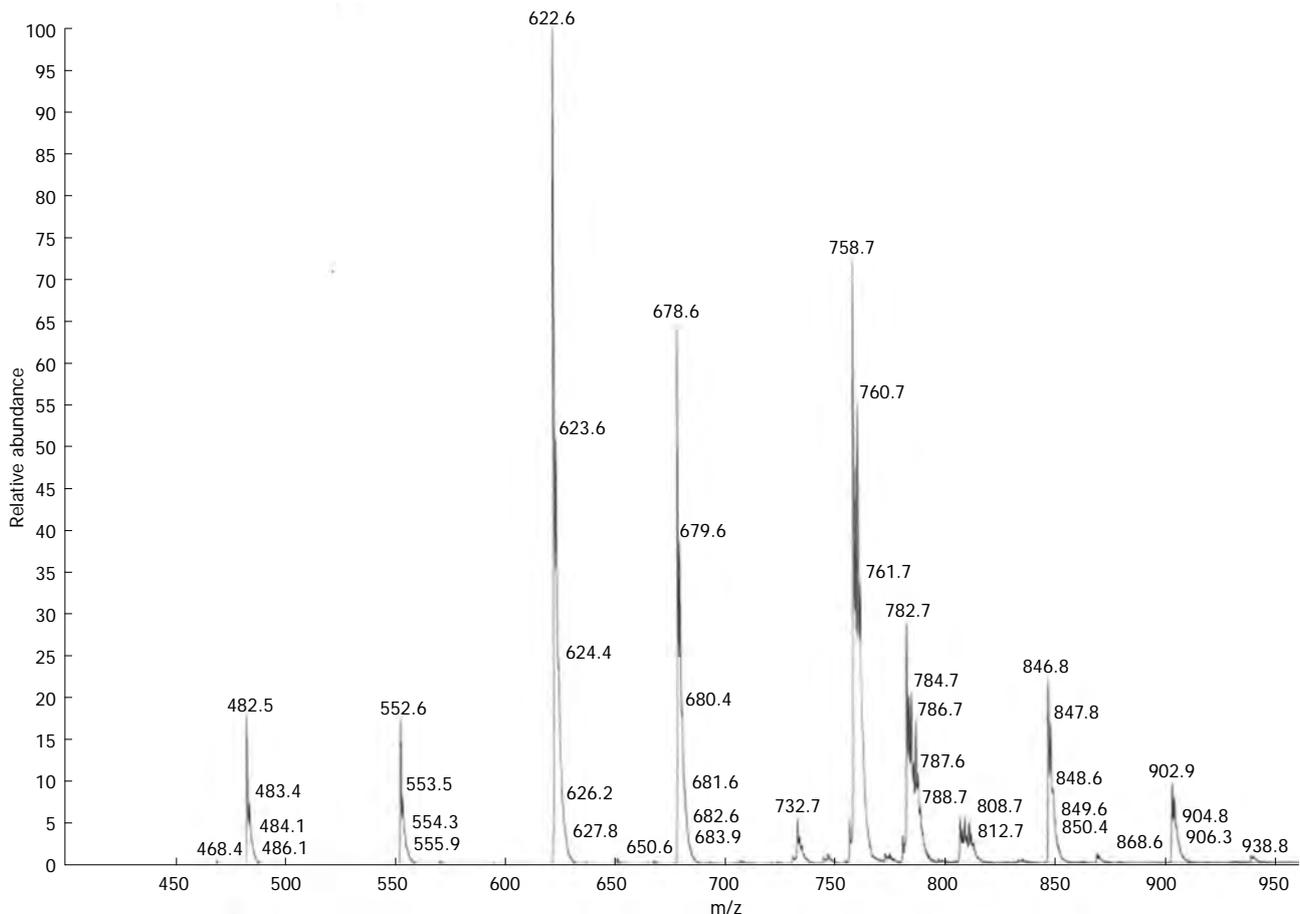


Figure 1 Electrospray mass spectrum of the total lipid extract from human bile of a patient with primary sclerosing cholangitis. For explanation of standards view Table 2.

tient groups were noted for serum AP levels at the time of ERC and for patient age (Table 1). Serum bilirubin concentrations, however, did not differ significantly between the groups.

### Biliary phospholipid analysis

The amounts of total phosphatidylcholine (PC) in relation to hepatic bile volume were compared between the four groups of patients (Table 3). They ranged between 47 and 22570  $\mu\text{mol/L}$  (median 2977  $\mu\text{mol/L}$ ). The highest biliary PC concentrations were noted in the controls (8030  $\pm$  1843  $\mu\text{mol/L}$ ), the lowest ones in SSC patients (2501  $\pm$  790  $\mu\text{mol/L}$ ) and CCC patients (1969  $\pm$  981  $\mu\text{mol/L}$ ) (overall  $P = 0.05$ , controls *vs* CCC and controls *vs* SSC,  $P < 0.05$ , respectively). In PSC patients, we found intermediate concentrations (6205  $\pm$  1465  $\mu\text{mol/L}$ ).

### Biliary PC species profiles

PC species in significant amounts were identified at molecular weights of 732, 734, 756, 758, 760, 782, 784, 786, 788, 804, 806, 808, and 810 Da. This corresponds to molecules with the following ratios of fatty acid (FA) carbon numbers to numbers of double bonds: 32:1, 32:0, 34:3, 34:2, 34:1, 36:4, 36:3, 36:2, 36:1, 38:7, 38:6, 38:5, and 38:4. Bile samples from the four patient groups showed a remarkably similar PC molecular species composi-

tion. Together constituting more than 50% of total PC, PC 34:1 and 34:2 represented in all cases the two most abundant PC species. DPPC (PC 34:0, 16:0-16:0) which is the most abundant PC species in pulmonary surfactant, represented only a very small percentage of biliary PC, ranging between 1.1% in controls and 3.6% in SSC patients (Table 4). Only minor intergroup differences were noted in biliary PC species patterns. These can be viewed in detail in Table 4 (for PC and LPC species, numbers of carbon atoms and double bonds of fatty acid side chains as well as molecular weights are indicated). Not even minor differences were found between PC species profiles in patients with PSC *vs* patients with SSC.

### LPC concentrations and LPC/PC ratios

Total LPC contents per bile volume ranged from 12.2  $\pm$  5.4  $\mu\text{mol/L}$  in CCC patients to 256  $\pm$  90  $\mu\text{mol/L}$  in the control group (overall,  $P = 0.02$ ; Dunn's post-test: no differences between single groups).

LPC species found in human hepatic bile had molecular weights of 496, 520, 522, and 524 Da, corresponding to 16:0, 18:3, 18:2, and 18:1, respectively. The distribution of different LPC species in the groups is shown in Table 4. Bile specimens obtained from patients with SSC contained relatively more LPC 496 (16:0) than those from patients with PSC and controls and relatively less LPC

**Table 3 Results of biochemical bile analyses of 41 human bile samples from four patient groups**

	Controls ( <i>n</i> = 11)	PSC ( <i>n</i> = 14)	SSC ( <i>n</i> = 10)	CCC ( <i>n</i> = 6) with PSC ( <i>n</i> = 2)	<i>P</i> value
Bile bilirubin (mg/dL)	41.6 ± 11.1 1 ND	11.3 ± 1.8 2 ND, 1 BLD	6.0 ± 1.7 1 BLD	14.0 ± 6.4	0.006; controls <sup>b</sup>
Bile protein (g/dL)	3.5 ± 0.9 1 ND	3.1 ± 0.5 3 ND	12.5 ± 9.5 1 BLD	3.7 ± 1.0	0.89
Bile cholesterol (mmol/L)	1.10 ± 0.32 2 BLD	0.53 ± 0.18 4 BLD	0.25 ± 0.07 4 BLD	0.31 ± 0.13	0.10
Bile total bile acids (mmol/L)	21.3 ± 3.1	17.4 ± 6.8	7.8 ± 3.1	5.9 ± 1.5	0.003; controls <sup>b</sup> , controls <sup>c</sup>
Bile total PC per volume (μmol/L)	8030 ± 1843	6205 ± 1465	2501 ± 790	1969 ± 981	0.005; controls <sup>a</sup> , controls <sup>c</sup>
Bile total LPC per volume (μmol/L)	256 ± 90	200 ± 97	91.3 ± 50.0	12.2 ± 5.4	Over-all 0.02; Dunn's post-test: NS
LPC/PC (molar ratio)	0.04 ± 0.01	0.09 ± 0.05	0.03 ± 0.01 <sup>1</sup>	0.008 ± 0.001	0.54
PC/bilirubin (molar ratio)	0.13 ± 0.07	0.35 ± 0.08	0.30 ± 0.07	0.24 ± 0.09	0.09
PC/protein [μmol/L/(g/dL)]	5131 ± 1918	3597 ± 1350	2782 ± 2241	725 ± 229	0.09
PC/cholesterol (molar ratio)	8.9 ± 2.6	14.0 ± 3.8	8.4 ± 1.4	6.2 ± 2.3	0.46
PC/bile acids (molar ratio)	0.40 ± 0.07	0.45 ± 0.06	0.34 ± 0.07	0.31 ± 0.09	0.45

<sup>1</sup>One value was excluded (outlier); for calculation of SEM, values below detection level were set at 0; ND: Not done (not sufficient material left for determination); BLD: Below limit of detection; NS: Not significant; PSC: Primary sclerosing cholangitis; SSC: Secondary sclerosing cholangitis; CCC: Cholangiocellular carcinoma. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs SSC; <sup>c</sup>*P* < 0.05 vs CCC.

**Table 4 Molar percentages of phosphatidylcholine and lysophosphatidylcholine species in bile from four patient groups**

	Molecular weight (Da)	Controls ( <i>n</i> = 11)	PSC ( <i>n</i> = 14)	SSC ( <i>n</i> = 10)	CCC ± PSC ( <i>n</i> = 6)	<i>P</i> value
PC molecular species						
32:1	732	2.8 ± 0.5	3.3 ± 0.4	9.2 ± 3.9	4.2 ± 0.7	0.036, controls vs SSC <sup>a</sup>
32:0	734	1.1 ± 0.1	1.4 ± 0.2	3.6 ± 1.2	1.5 ± 0.2	0.029, controls vs SSC <sup>a</sup>
34:3	756	2.2 ± 0.3	2.7 ± 0.2	2.7 ± 0.4	2.0 ± 0.4	0.19
34:2	758	33.7 ± 1.1	28.5 ± 0.7	25.8 ± 3.1	29.8 ± 0.2	0.017, controls vs PSC <sup>c</sup>
34:1	760	22.8 ± 0.9	22.1 ± 0.7	22.2 ± 0.9	24.1 ± 1.3	0.55
36:4	782	10.5 ± 0.6	10.3 ± 0.7	10.1 ± 1.8	8.6 ± 0.5	0.24
36:3	784	8.8 ± 0.3	10.0 ± 0.4	8.4 ± 0.8	7.1 ± 0.7	0.013, CCC vs PSC <sup>c</sup>
36:2	786	7.3 ± 0.4	9.0 ± 0.6	8.1 ± 0.9	9.3 ± 0.9	0.13
36:1	788	3.3 ± 0.3	3.5 ± 0.3	3.7 ± 0.4	3.2 ± 0.4	0.65
38:7	804	0.8 ± 0.2	0.9 ± 0.3	0.4 ± 0.3	0.2 ± 0.1	0.10
38:6	806	2.6 ± 0.2	3.3 ± 0.3	2.5 ± 0.5	6.3 ± 2.5	0.05
38:5	808	2.1 ± 0.1	2.7 ± 0.3	1.6 ± 0.4	2.0 ± 0.3	0.09
38:4	810	1.9 ± 0.2	2.3 ± 0.3	1.8 ± 0.4	1.9 ± 0.3	0.51
LPC molecular species						
16:0	496	59.2 ± 7.0	56.0 ± 5.8	85.0 ± 5.2	58.9 ± 7.0	0.05, controls vs SSC <sup>a</sup> , PSC vs SSC <sup>b</sup>
18:2	520	21.2 ± 6.0	12.6 ± 4.0	0.9 ± 0.9	30.0 ± 9.0	0.02, stones vs SSC <sup>c</sup> , CCC vs SSC <sup>b</sup>
18:1	522	9.7 ± 2.3	16.0 ± 2.9	7.4 ± 2.6	4.0 ± 4.0	0.08
18:0	524	9.9 ± 5.4	15.5 ± 2.3	6.8 ± 2.2	7.2 ± 7.2	0.06

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs secondary sclerosing cholangitis (SSC) group; <sup>c</sup>*P* < 0.05 vs primary sclerosing cholangitis (PSC). CCC: Cholangiocellular carcinoma; PC: Phosphatidylcholine; LPC: Lysophosphatidylcholine.

520 (18:3) than those from patients with CCC and from controls.

Since the cytotoxic effect of LPC on the bile duct mucosa is likely to be alleviated by the presence of PC<sup>[23]</sup>, it was interesting to examine the LPC/PC ratios in bile. Except for two bile specimens obtained from PSC patients and one bile specimen from a SSC patient (LPC/PC ratios: 0.25, 0.64 and 10.5), the biliary LPC/PC ratios were remarkably low in the other patients, LPC accounting for 4.2% ± 1.8% of total PC (mean ± SE). LPC/PC ratios were not different between the four groups.

In order to determine the extent of potential reflux of pancreatic juice in the bile specimens, pancreatic lipase activity was quantified in the samples. In 31 of 41 specimens examined, pancreas lipase activity could be de-

termined. Its average in these specimens was 266 ± 95.3 U/L. Interestingly, no significant correlation was found between pancreas lipase activity in bile and LPC/PC ratios (*P* = 0.66, *r* = 0.08), suggesting that bile LPC in the examined patients originated more likely from a different source.

#### Ratios of phospholipids to other bile components

As shown in Table 3, PC or LPC/bilirubin, PC or LPC/protein, PC or LPC/cholesterol as well as PC or LPC/total bile salt ratios in hepatic bile did not differ between controls and patients with PSC, SSC, or CCC.

#### Bacterial cultures and biliary phospholipids

At certain concentrations, bacteria in hepatic bile are

known to lead to the degradation of protective biliary PC and subsequent increase in the amounts of potentially cytotoxic LPC<sup>[24]</sup>. Thereby, they might cause chronic irritation of the bile duct mucosa and subsequently fibrosis. The 41 patients included in this study were divided into two groups according to the amount of bacterial growth in their bile. Group 1 comprised patients with no or only scarce amounts of bacteria and/or *Candida* organisms, while group 2 comprised patients with at least moderate amounts of bacteria in their bile specimens. According to this classification, 24 of 40 patients (no microbiological results available in one of the CCC patients) belonged to group 1. Among the bacterial species identified were mainly *Enterococcus faecalis* and *Enterococcus faecium* as well as *Escherichia coli*. Other species identified were *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Raoultella planticola*. Interestingly, biliary LPC/PC ratios did not differ significantly between the two groups.

## DISCUSSION

The main hypothesis of the present study was that, according to the “toxic bile” concept, alterations in biliary phospholipid concentrations and percentual distribution of species might play a role in the process of chronic inflammation and subsequent fibrosis in sclerosing cholangitis, such as PSC and SSC. Based on this hypothesis, we expected to find a biliary phospholipid imbalance with a lack of presumably cytoprotective PC and an abundance of presumably cytotoxic LPC in bile specimens from patients with sclerosing cholangitis.

To our knowledge, no data on MS of PC and LPC species patterns in human hepatic bile from patients with PSC and SSC have been published thus far. Most previous studies on biliary phospholipids have been performed using samples of gall bladder bile acquired during surgery in patients with gall stones<sup>[25-27]</sup>.

Compared to conventional methods of phospholipid analysis (*e.g.*, thin layer chromatography, derivatization, HPLC, and gas chromatography), electrospray-ionization (ESI)-tandem mass spectrometry (MS/MS) has several advantages since it has a high basic sensitivity for the detection of phospholipids (analyte concentrations between 0.1 and 50 pmol/ $\mu$ L), and since the specificity of MS/MS scan modes enables direct analysis of crude lipid extracts<sup>[28]</sup>. Thus, as little as 1  $\mu$ L of bile from every patient was sufficient for phospholipid analyses in the present study.

Further, for the analysis of bile in respect to potential secondary alterations of bile ducts, intrahepatic bile is more reliable than gall bladder bile. However, obtaining bile specimens during ERC has some drawbacks. Mostly, it is not justifiable from an ethical position to obtain specimens from completely healthy controls *via* ERC, since the procedure can cause complications, such as post-ERC pancreatitis, which is - of course - no different for patients in surgery. This is why we selected mainly

choledocholithiasis patients without signs of chronic inflammation as the controls in this study. However, lithogenic bile is known to show an imbalance between cholesterol, bile salts, and phospholipids. The primary pathophysiological defect in cholesterol gallstone disease is hypersecretion of hepatic cholesterol into bile with less frequent hyposecretion of bile salts and/or phospholipids<sup>[7]</sup>. For this reason, all the above-mentioned bile compounds were assessed in the present study.

The amount of total PC in relation to bile volume differed significantly between the controls and patients with SSC or CCC, with the latter two groups displaying lower concentrations (overall  $P = 0.005$ , controls *vs* SSC patients or CCC patients  $P < 0.05$ , respectively). The interpretation of this result is challenging since it cannot be ruled out that a few of the bile samples might have been diluted by contrast medium. However, the cases in which bile cannot be aspirated through the ERC-catheter before intervention are usually rare according to our experience, and we took care to first aspirate the contrast medium in such a case, and to discard it before obtaining the bile sample in a second syringe. Unfortunately, it was not indicated in the databank in which cases specimens were obtained without prior injection of contrast medium. Reduced biliary PC concentrations might be caused by lack or malfunction of biliary phospholipid transporters. Another reason for which the evaluation of total PC and LPC per bile volume might be hampered is that a high degree of cholestasis (with high serum levels of AP and bilirubin) can go along with low concentrations of bilirubin and the other analytes in bile. Bilirubin levels in serum from patients with SSC or CCC exceeded those of the other groups, albeit not significantly. AP levels in serum from patients with SSC were significantly higher than those in serum from controls. This is why we feel that - given the small sample sizes in the present study - the fact that we found lower levels of PC and LPC per bile volume should not be overrated. Yet we think that if our results can be confirmed in further studies with larger sample numbers and matched samples, a diagnostic tool might thereby be established. For such a future study, it would be helpful to take a note of the mode of bile acquisition in every included patient.

Importantly, the difference observed between the groups concerning total biliary PC per bile volume could not be reproduced when the groups were compared for the biliary PC/bile salt ratio, the reason for which might be dilution, as indicated above. The biliary PC/bile salt ratio was assumed to be of special pathophysiological importance since it may be indicative of a potential imbalance between factors protecting cholangiocytes and those harming cholangiocytes. Thus, before starting the study, we had hypothesized that PC-to-bile salt ratios in bile would be reduced in patients with sclerosing cholangitis *vs* controls. Our hypothesis was also due to recently published data showing that nonanastomotic strictures after liver transplantation were present more often in patients with low biliary phospholipids/bile salt ratios

than in patients with high biliary phospholipids/bile salt ratios<sup>[29]</sup>. When planning another study on this subject, it would be important to include more patients, and to not only focus on total bile salts as a reference parameter, but to also quantitate different bile salts with variable effects on cholangiocytes. Such an approach may also help to attenuate the intake of UDCA by many patients as a confounder.

Further, no major differences were noted in the biliary LPC/PC ratios and, most interestingly, in the LPC and PC species patterns in bile between the groups. As the distribution of biliary PC and LPC species was not relevantly changed in sclerosing cholangitis, we suggest that it plays no major role in the pathogenesis of the disease; based on our study, PC and LPC species patterns cannot be used as diagnostic tools to differentiate between PSC and SSC.

What is the role of LPC in bile? Remarkably, this study confirms that in normal bile, LPC is just a minor constituent. Analysis of the data for all 41 patients of the study together showed that total LPC accounted for less than 5% of total PC, after the exclusion of three patients with exceedingly high ratios, who were treated as outliers. In line with previous studies by other authors, our results revealed only traces of LPC in hepatic bile from “controls”<sup>[30]</sup>. LPC can be derived from PC by hydrolysis within the bile ducts. Nakano *et al.*<sup>[24]</sup> found that most bacterial strains isolated from bile possess both phospholipase A1 and A2 activity. Shimada *et al.*<sup>[27]</sup> showed in their study that patients with an anomalous pancreaticobiliary ductal junction have considerable amounts of LPC in their intrahepatic bile, which could not be correlated with concentrations of bacteria in bile, but with phospholipase A2 activity from refluxing pancreatic juice.

Although the four groups did not differ with respect to the biliary LPC/PC ratios in the present study, a few bile specimens displayed high biliary LPC/PC ratios. Retrospective analysis of these patients showed high lipase concentrations and/or abundant bacterial growth in some, but not in all, cases; some patients also showed abundant growth of bacteria in bile and/or high lipase concentrations with very low LPC/PC ratios. Again, the small numbers of subgroups might disguise potentially important differences here. Furthermore, most bile specimens with abundant bacterial growth contained predominantly gram-positive cocci. The latter have been previously shown to produce just minor amounts of phospholipases<sup>[24]</sup>. Considering all the data, it is still reasonable to suggest that recurrent bacterial cholangitis caused by certain bacterial strains in patients with a prior diagnosis of sclerosing cholangitis might lead to the aggravation of disease activity *via* increased LPC/PC ratios in bile, even though this could not be proven in the present study.

Data have recently been published on the lack of PC in the bile from patients with CCC<sup>[31]</sup>. Although total biliary PC contents per volume in the specimens from CCC patients in the present study were the lowest compared to the other groups, ratios of PC contents to any other

biliary compound did not differ between groups. Since the number of CCC patients was below ten in both the above-mentioned study and ours, further studies with larger sample numbers are necessary to determine whether patients with CCC indeed have lower biliary PC contents. This would be of major importance as there is still no diagnostic tool available for early diagnosis of CCC, especially in patients with known PSC.

PC and LPC species patterns in human bile were quite similar to those found in intestinal mucus in a previous study<sup>[32]</sup>. Unlike the case with pulmonary surfactant, biliary PC molecules with fatty acids containing at least one double bond clearly overweigh those with saturated fatty acid side chains. The fact that PC and LPC species patterns were remarkably similar between the groups supports the idea that biliary PC and LPC form a highly preserved system that cannot easily be changed by external influences. Previous reports indicating that bile of various animal species displayed a very similar phospholipid molecular species composition are also supportive of this notion<sup>[33]</sup>.

In conclusion, we showed that electrospray MS/MS is a very convenient method for bile phospholipid analysis in very low volume samples. Surprisingly, there were no major differences concerning the biliary PC and LPC species profiles between patients with PSC, SSC, CCC and controls. Yet our data can serve as an incentive and reference for further studies using the same methods for larger groups of patients or other disease conditions. Also, the lower total PC and LPC concentrations found in bile from patients with SSC and CCC compared to controls might be of diagnostic importance, if the results were certified in further studies with larger sample numbers and after exclusion of dilutional effects.

## COMMENTS

### Background

Primary sclerosing cholangitis (PSC) and secondary sclerosing cholangitis (SSC) are progressive diseases where chronic inflammation leads to scarring and strictures of the bile ducts. The process eventually results in liver cirrhosis and makes liver transplantation necessary. The two diseases have very similar features, but their pathogeneses seem to be different even though they are not yet fully known. In SSC, the afflictions of the bile ducts originate from a trigger in the patient's history, like *e.g.*, hypoxemia. SSC also progresses more rapidly than PSC. Biliary phospholipid transporter defects have been shown to lead to bile duct fibrosis in mice. Phosphatidylcholine (PC) is thought to have protective properties in bile. These facts led to the hypothesis that a deficiency of PC or an imbalance of its single species, or a relative abundance of potentially toxic lysophosphatidylcholine (LPC) could play a role in the pathogenesis of SSC and more significantly PSC. Such a finding could lead to the development of novel therapeutic options.

### Research frontiers

Due to its unfavorable disease course, lack of thorough understanding of the underlying pathophysiology, and absence of effective medical therapy, sclerosing cholangitis has become a subject of increasing scientific interest. In contrast to PSC, SSC has not been described at all until recently, so research in this field is breaking new ground. Research has been published on the relation between changes in biliary phospholipids and bile duct strictures after liver transplantation, but none on biliary PC and LPC species in PSC as well as SSC. The “toxic bile concept” - the theory which states that imbalances in the composition of bile could lead to chronic bile duct inflammation and destruction - remains relevant,

as a therapy would likely arise from the identification and regulation of these potential imbalances. Such a therapy would be effective regardless of whether these imbalances were primary or secondary factors of disease activity.

### Innovations and breakthroughs

Even though the data presented here have no direct implications for diagnosis or therapy, they can serve as motivation for future studies in the field using the very convenient and resource-economic method of nano-electrospray ionization mass spectrometry for bile phospholipid analysis. In this study, data on PC and LPC profiles in intrahepatic human bile in SSC are published for the first time.

### Applications

The method of nano-electrospray ionization mass spectrometry of bile phospholipids can be used for the examination of larger patient groups. Biliary phospholipid composition in cases of cholangiocellular carcinoma and after liver transplantation might be of special interest. As little as 1  $\mu$ L of bile is needed for analysis of all PC and LPC species.

### Terminology

PC is not only an essential component of biological membranes, but also of a wide array of functionally important body fluids like pulmonary surfactant, gastric and intestinal mucus, synovial fluid, peritoneal fluid and bile. It is a glycerophospholipid featuring a choline head group and two fatty acid side chains. The special biophysical qualities of these zwitter-ionic molecules depend on the composition of these fatty acid side chains. For example, PC in pulmonary surfactant has mostly saturated fatty acids, while in intestinal mucus - having to meet completely different functional challenges - PC almost always contains at least one unsaturated fatty acid side chain. This is why the exact composition of PC species of a fluid like bile is so important. LPC is produced from PC by partial hydrolysis, resulting in removal of one fatty acid side chain.

### Peer review

This manuscript is quite interesting, with a good methodology. There are not many patients, but due to ERC and rare etiologies, these could be enough. The authors took into account their limitations and made a clear discussion. This small study about the pathophysiology of sclerosing cholangitis is well designed and presented.

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## Extracorporeal continuous portal diversion plus temporal plasmapheresis for “small-for-size” syndrome

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### Abstract

**AIM:** To investigate the effect of plasmapheresis *via* the portal vein for “small-for-size” syndrome (SFSS) aided by extracorporeal continuous portal diversion (ECPD).

**METHODS:** Extensive or total hepatectomy in the pig is usually adopted as a postoperative liver failure (PLF) or SFSS model. In this study, animals which underwent 85%-90% hepatectomy were randomized into either the Systemic group ( $n = 7$ ) or the Portal group ( $n = 7$ ). In the Systemic group, all pigs received temporal plasmapheresis (PP) *via* the extracorporeal catheter circuit (systemic to systemic circulation) from 24 to 30 h post-hepatectomy (PH); in the Portal group, all pigs received ECPD to divert partial portal vein flow (PVF) to the systemic circulation after hepatectomy, then converted to temporal PP from 24 to 30 h PH, and subsequently converted to ECPD again until 48 h PH. In the Portal group, the PVF was preserved at 3.0-3.3 times that of the baseline value, similar to that following 70% hepatectomy, which was regarded as the optimal PVF to the

hypertrophic liver remnant. At 48 h PH, all pigs were re-opened and the portal vein pressure (PVP), PVF, and HAF (hepatic artery flow) were measured, and then diversion of the portal venous flow was terminated. After 1 h the PVP, PVF, and HAF were re-measured. The portal hemodynamic changes, liver injury, liver regeneration and bacterial/lipopolysaccharide (LPS) translocation were evaluated in the two groups.

**RESULTS:** The PVP in the Portal group was significantly lower than that in the Systemic group during the time period from 2 to 49 h PH ( $P < 0.05$ ). Serum alanine aminotransferase (ALT), total bilirubin (TB) and ammonia were significantly reduced in the Portal group compared with the Systemic group from 24 to 48 h PH ( $P < 0.05$ ). The Portal group may have attenuated sinusoidal endothelial injury and decreased the level of HA compared with the Systemic group. In the Systemic group, there was significant sinusoidal dilation, hydropic changes in hepatocytes and hemorrhage into the hepatic parenchyma, and the sinusoidal endothelial lining was partially destroyed and detached into the sinusoidal space. CD<sub>31</sub> immunostaining revealed significant destruction of the endothelial lining. In the Portal group, there was no intraparenchymal hemorrhage and the sinusoidal endothelial cells and hepatocytes were well preserved. CD<sub>31</sub> immunostaining was mild which indicated less destruction of the endothelial lining. HA was significantly decreased in the Portal group compared with the Systemic group from 2 to 48 h PH. The rate of liver remnant regeneration was elevated, while apoptosis was attenuated in the Portal group compared with the Systemic group. Thymidine kinase activity was much higher in the Portal group than in the Systemic group at 48 h PH. The PCNA index was significantly increased and the apoptotic index was significantly decreased in the Portal group compared with the Systemic group. Bacterial translocation and endotoxin, as well as the inflammatory response, were significantly attenuated in the Portal group compared with the Systemic group. LPS, tumor necrosis factor- $\alpha$  and interleukin-6 levels were all significantly decreased in the Portal

group compared with the Systemic group from 24 to 48 h PH, while bacterial DNA level was significantly decreased from 2 to 48 h PH.

**CONCLUSION:** PP plus ECPD *via* the portal vein can attenuate toxic load and hyperperfusion injury, and should be undertaken instead of PP *via* the systemic circulation in SFSS or PLF.

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**Key words:** Small-for-size syndrome; Postoperative liver failure; Extracorporeal portal diversion; Plasmapheresis; Hepatectomy

**Core tip:** Plasmapheresis (PP) and other artificial liver support (ALS) modalities have been used to treat postoperative liver failure (PLF) and “small-for-size” syndrome (SFSS). However, these modalities did not result in a significant improvement in survival. It is thought that these modalities cannot relieve portal hypertension, thus are inefficacious. This study demonstrated that ECPD plus temporal PP *via* the portal vein can not only dynamically turn the portal flow to the systematic circulation and attenuate portal overflow injury, but also reduces toxic load. This technique should be undertaken instead of PP or ALS *via* the systemic circulation in SFSS or PLF, and shows potential for clinical application.

Hou P, Chen C, Tu YL, Zhu ZM, Tan JW. Extracorporeal continuous portal diversion plus temporal plasmapheresis for “small-for-size” syndrome. *World J Gastroenterol* 2013; 19(33): 5464-5472 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5464.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5464>

## INTRODUCTION

Plasmapheresis (PP) and other plasma purification modalities have been used in the past to treat postoperative liver failure (PLF) and “small-for-size” syndrome (SFSS) following extensive liver resection and living donor liver transplantation (LDLT)<sup>[1-4]</sup>. However, none of these modalities has resulted in a significant improvement in survival. Currently, it is deemed that PP and other modalities *via* systemic circulation access adopted in clinical practice can decrease toxin load and improve serum biochemistry, but do not relieve portal hypertension or hyperperfusion, which results in significant harm to sinusoidal endothelial cells, liver function and intestinal barrier function and is regarded as the determining pathogenesis of PLF and SFSS following subtotal or critical hepatectomy<sup>[5-8]</sup>. This study aims to investigate the effects of temporal PP *via* portal vein access with the aid of extracorporeal continuous portal diversion (ECPD) in SFSS and PLF compared with temporal PP *via* systemic circulation access in a porcine model.

## MATERIALS AND METHODS

### Animal model of PLF and SFSS

Fourteen male Bama miniature pigs (15-20 kg) were obtained from the Pig and Poultry Production Institute (Guangxi Autonomous Region, China). The pigs were raised from a closed herd and kept under strict quarantine. All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH). All animals in this study were treated humanely in accordance with institutional and national guidelines for the ethical treatment of animals.

All 14 animals, were anesthetized by initial sedation with a deep intramuscular injection of ketamine (15-20 mg/kg) and chlorpromazine (6-8 mg/kg) 15 min after the administration of atropine (0.01 mg/kg), then underwent 85%-90% hepatectomy (left tri-lobe and partial right posterior lobe resection) with less than 60 mL blood loss and no hepatic pedicle occlusion, according to a previously described protocol<sup>[6,9]</sup>. First, ultrasonic flow probes were connected to a flow meter (Transonic Systems INC. TS420, NY, United States) to measure hepatic artery flow (HAF) and portal vein flow (PVF). Second, two 11.5-Fr dual-lumen catheters (Hanahao, Tyco Healthcare, Tianjin, China) were introduced into the upper vena cava through the internal jugular vein and the portal vein before hepatectomy.

### Study protocol

Extensive or total hepatectomy in the pig is usually adopted as a model of acute liver failure<sup>[10]</sup>. All animals which underwent 85%-90% hepatectomy were randomized into either the Systemic group ( $n = 7$ ) or the Portal group ( $n = 7$ ). In the Systemic group, all pigs received temporal PP *via* the extracorporeal catheter circuit (systemic to systemic circulation) from 24 to 30 h post-hepatectomy (PH); in the Portal group, all pigs received ECPD to divert partial PVF to the systemic circulation after hepatectomy, then converted to temporal PP from 24 to 30 h PH, and subsequently converted to ECPD again until 48 h PH. In the Portal group, the PVF was preserved at 3.0-3.3 times that of the baseline value, similar to that following 70% hepatectomy, which was regarded as the optimal PVF to the hypertrophic liver remnant<sup>[5]</sup>. At 48 h PH, all pigs were re-opened and the portal vein pressure (PVP), PVF, and HAF were measured, then diversion of the portal venous flow was terminated. After 1 h, the PVP, PVF, and HAF were re-measured. The portal hemodynamic changes, liver injury, liver regeneration and bacterial/lipopolysaccharide (LPS) translocation were evaluated in the two groups.

### Postoperative management

At the end of surgery, one dose of 375 mg penicillin/streptomycin was given intramuscularly to all pigs. This dose was repeated daily every morning until the pigs were euthanized. Each pig was allowed access to food and water ad libitum in the postoperative phase, and they

were monitored postoperatively until euthanized at 49 h PH. The pigs’ systemic arterial pressure was monitored throughout the experiment. Food and water intake and serum glucose levels were evaluated at each postoperative assessment. The liver remnant was removed, weighed, and sampled, and the animals were euthanized.

### Plasma extraction

During each extraction, 200-300 mL plasma was obtained from 6 donor pigs *via* the internal jugular vein catheter and immediately frozen at -20 °C.

### PVF, HAF, and PVP measurement

PVF, HAF, and PVP were measured in both groups at several time points during the procedure: at laparotomy, at 1 h PH, 24 h PH, and 48 h PH. At 48 h PH, the pigs were re-opened, the ECPD was stopped, and the PVP, HAF and PVF were re-measured.

### Blood and serum analysis

Blood samples at pre-operation, 2, 24, 30, and 48 h PH were collected and analyzed. The serum levels of alanine aminotransferase (ALT), total bilirubin (TB) and ammonia were determined in these samples. Increased serum level of hyaluronic acid (HA), which is chiefly eliminated in the hepatic sinusoidal endothelium, indicated sinusoidal endothelial damage<sup>[11,12]</sup>. HA in serum samples was measured by radiometric assay using the Pharmacia HA test (Shanghai Hua Yi Scientific, Inc., Shanghai, China) at pre-operation, 2 h PH, 24 h PH and 48 h PH. Thymidine kinase (TK) activity is the index of hepatic regeneration. Serum TK activity was measured with the Liaison TK assay (DiaSorin, Inc., Stillwater, MN, United States) at pre-operation, 2 h PH, 24 h PH and 48 h PH<sup>[13,14]</sup>.

### Histological analysis

Hepatic tissue was sampled at 1 h PH. Each biopsy sample was divided into 2 specimens. The tissue specimens for electron microscopy were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.3). The other set of samples were preserved in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Then, 4- $\mu$ m-thick sections were immunostained with porcine anti-CD31 antibody (Serotec, Oxford, United Kingdom) to evaluate the microstructural integrity of the hepatic sinusoid<sup>[15,16]</sup>. The animals were sacrificed at 49-50 h PH. The liver was excised after laparotomy, then weighed and processed. The hepatic tissue was sampled again, preserved in 10% neutral-buffered formalin and embedded in paraffin for proliferating cell nuclear antigen (PCNA) immunostaining and *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) examination.

The PCNA expression was detected by immunostaining using monoclonal anti-PCNA-antibody (DAKO) (Shanghai Hua Yi Scientific, Inc., Shanghai, China). The rate of increase of liver volume was evaluated by the following equation: The rate of increase = liver volume at sacrifice/

estimated remnant liver volume at operation  $\times$  100%.

Liver samples at 48 h PH were stained for PCNA. PCNA is a stable cell-cycle nuclear protein. The rate of DNA synthesis correlates with proliferation of the cells. Data were expressed as the percentage of hepatocytes stained with PCNA. The percent of PCNA-stained hepatocytes in the total cells per 10 high-power fields was calculated and compared between the two groups.

Three-micrometer-thick sections were stained with hematoxylin and eosin and analyzed by TUNEL using an *in situ* apoptosis-detection kit (Jiamei Biotech Co. Ltd., Shenzhen, China) following the manufacturer’s instructions. The percent of apoptotic cells in the total cells in each high-power field was measured and compared between the Portal group and the Systemic group. Ten consecutive high-power fields were calculated at  $\times$  400 magnification for each pig.

### Measurement of LPS levels

LPS levels in serum samples were measured by the quantitative chromogenic limulus amoebocyte lysate test (Yihua BioScience Ltd. Shanghai, China) according to the manufacturer’s instructions. All samples were tested in duplicate and read at 405 nm<sup>[17]</sup>.

### Bacterial translocation

Total bacterial quantification was accomplished by DNA isolation and real-time polymerase chain reaction (PCR). DNA was isolated from blood using the Fast DNA Spin Kit (Cat. 69506; Qiagen, United States) according to the manufacturer’s instructions. Subsequently, total bacterial quantification was performed with 16S rRNA gene-targeted primers. The sequences of the universal primers were as follows: 5’TTCCGGTTGATCCTGCCGGA3’ forward and 5’GGTTACCTTGTTACGACTT-3’ reverse<sup>[18,19]</sup>. The serially diluted genomic DNA of selected bacterial isolates was used as a real-time PCR positive control for total bacterial quantification. Bacterial counts are expressed as Log<sub>10</sub> cells per gram tissue (cells/g).

### Quantification of serum cytokine levels

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1) and IL-6 levels in serum were measured using enzyme-linked immunosorbent assays (ELISAs) following the manufacturer’s instructions (Jingmei Biotech Co. Ltd., Shenzhen, China). All samples were tested in triplicate and read at 490 nm in a thermomax microplate reader.

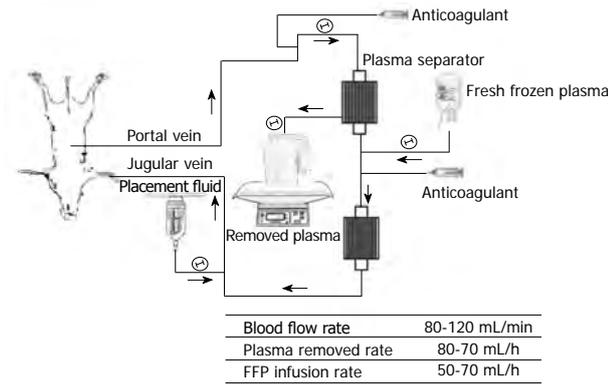
### Statistical analysis

All variables were expressed as mean  $\pm$  SD and compared with the Student’s *t* test using PASW Statistics 18 software (SPSS Inc., Chicago, IL, United States). *P* values of less than 0.05 were considered significant.

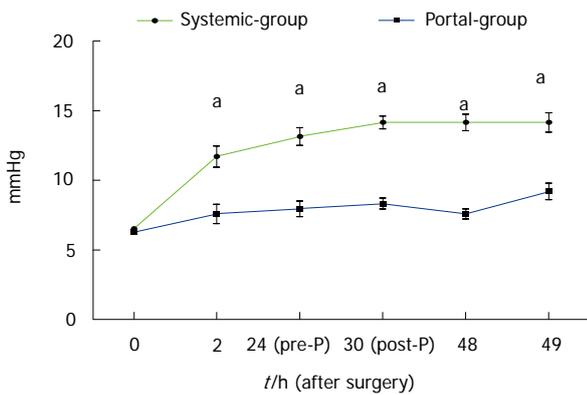
## RESULTS

### Arrangement of the experiment and the hemodynamic studies

Extracorporeal continuous portal diversion (ECPD) plus



**Figure 1** Flow diagram of the extracorporeal circuit indicating the plasmapheresis and conditions of slow plasma exchange. The portal venous blood was aspirated through the portal catheter and into tubing connected to a centrifugal pump immediately post-hepatectomy (PH), and then passed through plasma-separation cartridges with a blood flow of 90-110 mL/min. From 24 h PH these pigs were converted to plasma exchange for 6 h. After that the blood was returned to the pig through a double-lumen catheter inserted into the internal jugular or subclavian vein, and then continued on extracorporeal continuous portal diversion until 48 h PH. FFP: Fresh frozen plasma.



**Figure 2** Portal vein pressure was significantly reduced in the Portal group compared with the Systemic group. Portal vein pressure in the Portal group was significantly lower than that in the Systemic group from 2 to 49 h post-hepatectomy. <sup>a</sup>*P* < 0.05 indicates Portal group vs Systemic group.

temporal plasmapheresis (PP) by the extracorporeal catheter circuit was established (Figure 1). In the Portal group, portal venous blood was aspirated through the portal catheter and into tubing connected to a centrifugal pump immediately PH. The portal venous blood first passed through the centrifugal pump and then through plasma-separation cartridges with a blood flow of 90-110 mL/min to preserve the pigs’ PVF per unit volume range of 3.0-3.3 times that of the baseline. From 24 h PH, these pigs were converted to plasma exchange or PP for 6 h. After plasma exchange, the blood was returned to the pig through a double-lumen catheter inserted into the internal jugular or subclavian vein, and then continued on ECPD until 48 h PH. In the Systemic group, the extracorporeal catheter circuit was established with systemic circulation to systemic circulation at 24 h PH. The systemic circulation blood was aspirated through the systemic circulation catheter and into tubing connected to a centrifugal pump, and the same plasma exchange as the above group was performed for

**Table 1** Hemodynamic parameters measured at baseline, immediately post-hepatectomy, 48 h post-hepatectomy and euthanasia (49 h post-hepatectomy)

	Systemic group	Portal group	P value
Body weight (kg)	22.4 ± 3.4	23.6 ± 3.6	0.910
Left trilobes (g)	381.2 ± 14.9	390.5 ± 15.8	0.840
ETL (g)	476.8 ± 18.4	487.0 ± 19.7	0.860
WRL (g)	412.1 ± 15.6	413.1 ± 20.4	0.790
ERL (g)	61.7 ± 3.8	63.8 ± 4.1	0.760
Proportion of ERL	12.8% ± 2.3%	13.1% ± 3.5%	0.870
Operation time (min)	110 ± 23	126 ± 28	0.450
Blood loss (mL)	41.7 ± 13.8	49.1 ± 16.1	0.730
PVF (mL/min per 100 g)			
BAS	61.9 ± 9.6	64.1 ± 10.6	0.945
2 h PH	431.8 ± 36.6	238.8 ± 29.3	0.002
48 h PH	220.3 ± 21.1	152.3 ± 21.6	0.014
49 h PH	214.3 ± 26.1	227.4 ± 27.6 <sup>1</sup>	0.674
HAF (mL/min per 100 g)			
BAS	19.4 ± 4.5	19.9 ± 4.1	0.921
2 h PH	6.1 ± 2.5	14.9 ± 2.5	0.003
48 h PH	7.9 ± 2.1	13.2 ± 4.2	0.002
EUT (49 h PH)	8.2 ± 2.4	11.6 ± 3.5	0.003
P/A			
BAS	3.1 ± 0.2	3.2 ± 0.2	0.843
2 h PH	70.8 ± 8.1	16.0 ± 3.1	0.000
48 h PH	27.8 ± 6.6	11.5 ± 1.8	0.002
EUT (49 h PH)	26.1 ± 4.9	19.4 ± 4.6 <sup>1</sup>	0.001

All flow values are reported in mL/min per 100 g hepatic tissue. The data are expressed as mean ± SD. Estimated total liver weight = Weight of left trilobes × 100/80. ETL: Estimated total liver weight; WRL: Weight of resected liver; ERL: Estimated residual liver weight; PH: Post-hepatectomy; BAS: Baseline; EUT: Euthanasia; P/A: Portal-to-arterial flow ratio; PVF: Portal vein flow. <sup>1</sup>Extracorporeal continuous portal diversion was stopped for 1 h.

6 h in all pigs from 24 to 30 h PH, and then stopped after the blood was returned to the pig.

The infusion plasma volume was equal to 1.3 times the plasma volume that had been removed per hour in each pig. The total exchanged plasma volume was 4000-5000 mL each time. The adequacy of anticoagulation was monitored by activated clotting time (ACT), and heparin was administered as required to maintain ACT levels greater than 250 s. Standard monitoring (ECG and arterial line for blood pressure, and Foley catheter for urine output) was performed for all pigs.

The characteristics of the hemodynamic studies are shown in Table 1. The evolution of hemodynamic parameters was measured at baseline, immediately PH, 48 h PH, and euthanasia (49 h PH). All flow values are reported in mL/min per 100 g hepatic tissue. The results showed that hemodynamic parameters such as PVF, HAF and P/A gradually reduced with time in the Systemic group and the Portal group. These hemodynamic parameters were significantly decreased in the Portal group compared with the Systemic group.

**PVP was significantly reduced in the Portal group compared with the Systemic group**

The changes in PVP in both groups are shown in Figure 2. The results showed that PVP in the Portal group was significantly lower than that in the Systemic group during the time period from 2 to 49 h PH (*P* < 0.05). These results

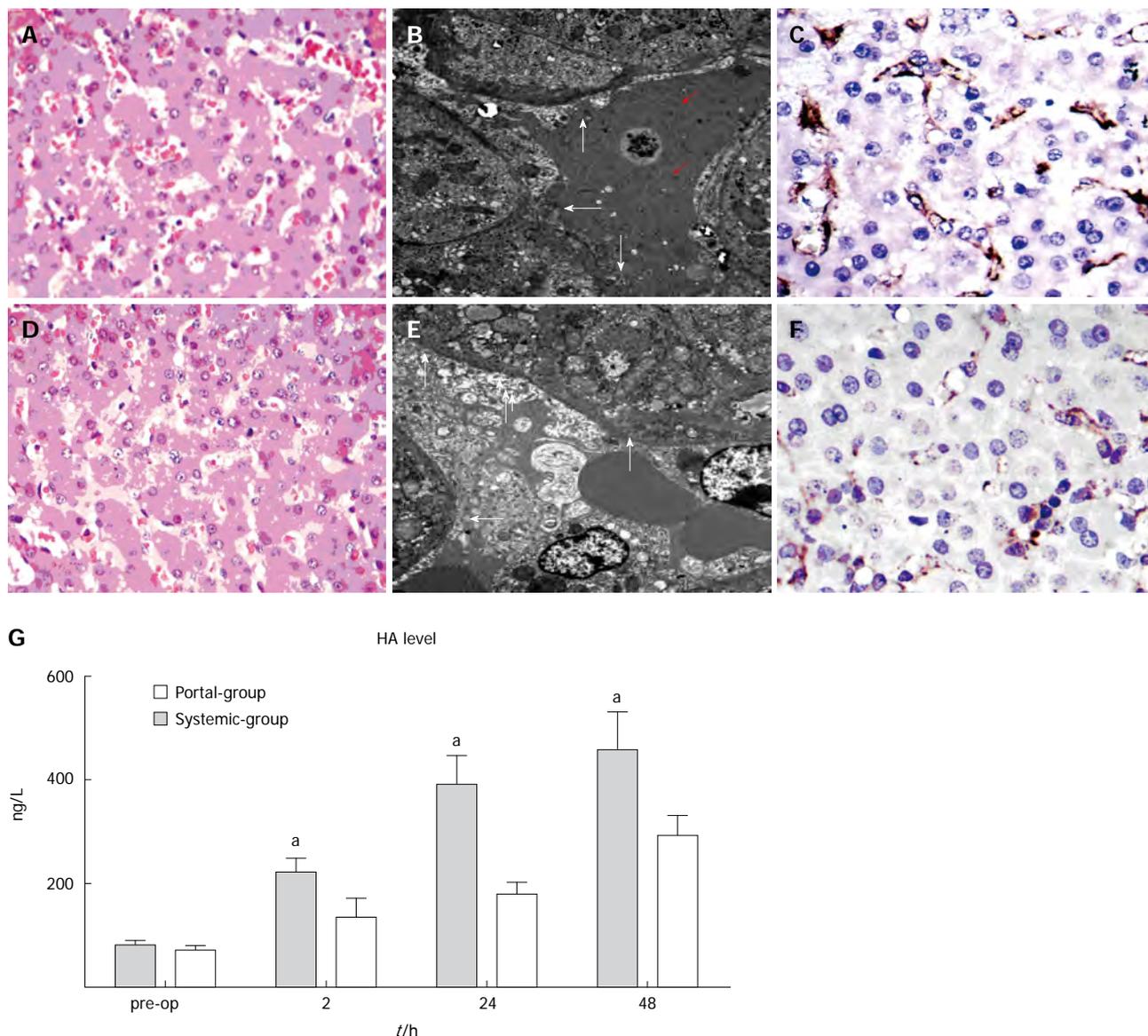


Figure 3 Portal group attenuated sinusoidal endothelial injury compared with the Systemic group. A, D: Hematoxylin and eosin staining ( $\times 400$  magnification) in the Systemic group and Portal group, respectively; B, E: Transmission electron microscopic (TEM) photographs ( $\times 6000$  magnification) in the Systemic group and Portal group, respectively; C, F: CD31 immunohistochemical staining of tissue samples ( $\times 400$  magnification) taken at 1 h post-hepatectomy (PH) in the Systemic group and Portal group, respectively; G: Serial changes in the level of hyaluronic acid (HA) in the two groups. <sup>a</sup> $P < 0.05$  indicates Portal group vs Systemic group.

suggested that PVP in the Portal group reduced much more than in the Systemic group. Thus, the Portal group had fewer complications in terms of portal hypertension compared with the Systemic group.

**Serum ALT, TB and ammonia were reduced in the Portal group compared with the Systemic group**

Levels of serum ammonia, ALT and TB collected serially during the follow-up period are shown in Table 2. The results showed that serum ALT, TB and ammonia were significantly reduced in the Portal group compared with the Systemic group from 24 to 48 h PH ( $P < 0.05$ ). In addition, serum ALT and ammonia in the Portal group were significantly improved, while TB remained the same after PP from 24 to 30 h PH ( $P < 0.05$ ). These results indicated that the Portal group may be better than the

Systemic group in improving liver function in PLF, and PP in the Portal group might enhance this protection of liver function.

**Portal group attenuated sinusoidal endothelial injury compared with the Systemic group**

In the Systemic group, there was significant sinusoidal dilation, hydropic changes in hepatocytes and hemorrhage into the hepatic parenchyma (Figure 3A). The sinusoidal endothelial lining was partially destroyed and detached into the sinusoidal space, accompanied by enlargement of the Disse’s spaces (red arrows) (Figure 3B). CD31 immunostaining revealed significant destruction of the endothelial lining (Figure 3C); whereas in the Portal group, no intraparenchymal hemorrhage was observed (Figure 3D), the sinusoidal endothelial cells and hepatocytes were

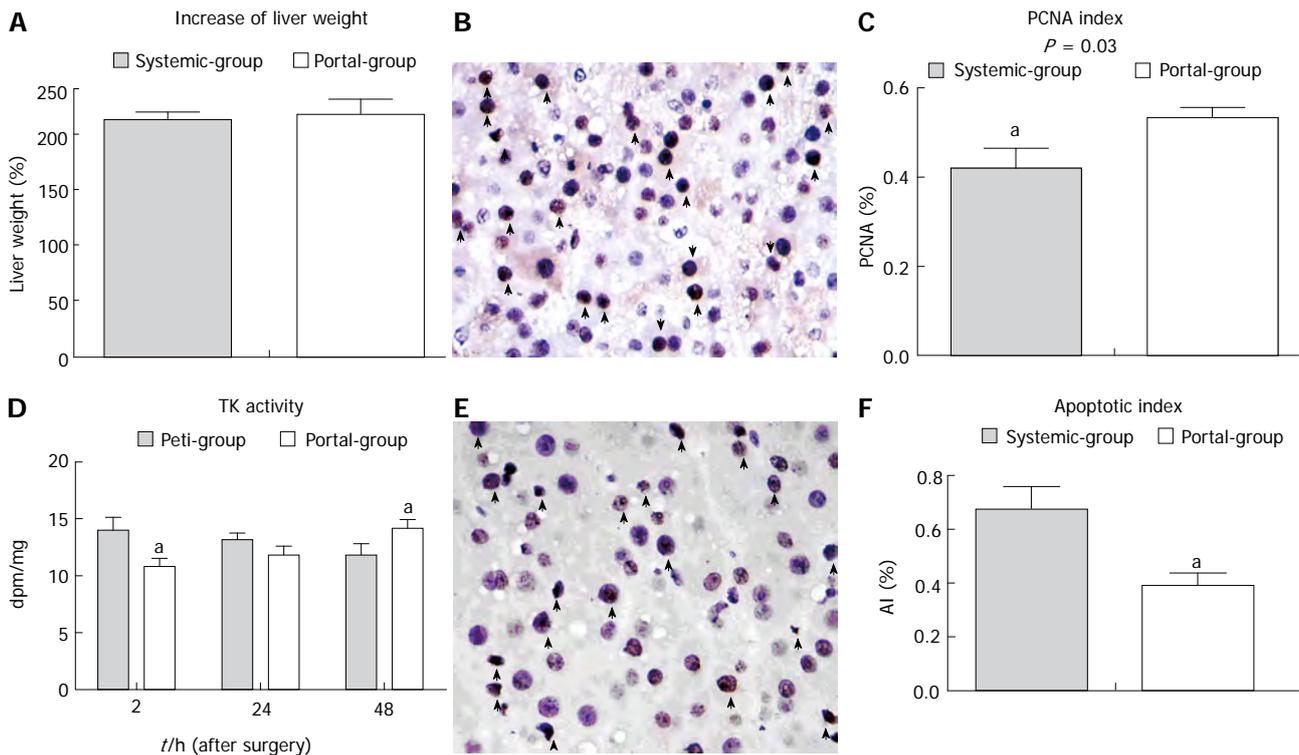


Figure 4 The rate of liver remnant regeneration was elevated and apoptosis was attenuated in the Portal group compared with the Systemic group. A: The rate of increase of liver volume in the two groups; B: Proliferating cell nuclear antigen (PCNA) staining in the liver remnant (arrows indicate stained positive cells × 400 magnification). A large number of transferase dUTP nick end labeling (TUNEL)-positive cells (arrows) in the liver remnant (× 400 magnification); C: Microphotometric evaluation of the PCNA index in PCNA-stained tissue at 48 h PH; D: The change in thymidine kinase activity in the two groups; E: TUNEL staining at 48 h PH; F: Microphotometric evaluation of the apoptotic index (AI) in TUNEL-stained tissue at 48 h PH. <sup>a</sup>*P* < 0.05 indicates a significant difference between the groups.

**Table 2 Serial change in serum ammonia, alanine aminotransferase and total bilirubin levels in the two groups**

	ALT (U/L)		TB (mmol/L)		Ammonia (μmol/L)	
	Systemic	Portal	Systemic	Portal	Systemic	Portal
Pre	45.2 ± 12.1	51.3 ± 15.5	17.3 ± 4.1	16.4 ± 5.5	158.4 ± 57.5	164.3 ± 46.2
2 h	67.2 ± 23.4	61.7 ± 26.1	19.5 ± 6.1	18.6 ± 6.3	239.6 ± 61.8	193.7 ± 47.0
24 h	129.7 ± 35.2	78.6 ± 24.5 <sup>1</sup>	45.9 ± 8.5	28.4 ± 5.7 <sup>1</sup>	345.2 ± 59.5	210.3 ± 67.7 <sup>1</sup>
30 h	101.5 ± 23.2	67.2 ± 16.4 <sup>1,2</sup>	36.4 ± 7.4	25.8 ± 5.1 <sup>1</sup>	217.4 ± 51.8	131.7 ± 37.4 <sup>1,2</sup>
48 h	118.6 ± 31.4	74 ± 29 <sup>1</sup>	58.4 ± 9.0	38.3 ± 7.1 <sup>1</sup>	254.3 ± 49.7	180.1 ± 54.5 <sup>1</sup>

<sup>1</sup>Indicates a significant difference between the two groups (*P* < 0.05); and <sup>2</sup>Indicates a significant difference in levels before and after Plasmapheresis (*P* < 0.05). ALT: Alanine aminotransferase; TB: Total bilirubin.

well preserved (arrow, Figure 3E), and CD31 immunostaining was mild which indicated less destruction of the endothelial lining (Figure 3F). Serial changes in the levels of HA in the two groups are shown in Figure 3G. HA was significantly decreased in the Portal group compared with the Systemic group from 2 to 48 h PH (*P* < 0.05). These results suggested that the Portal group may have attenuated sinusoidal endothelial injury and decreased HA level compared with the Systemic group.

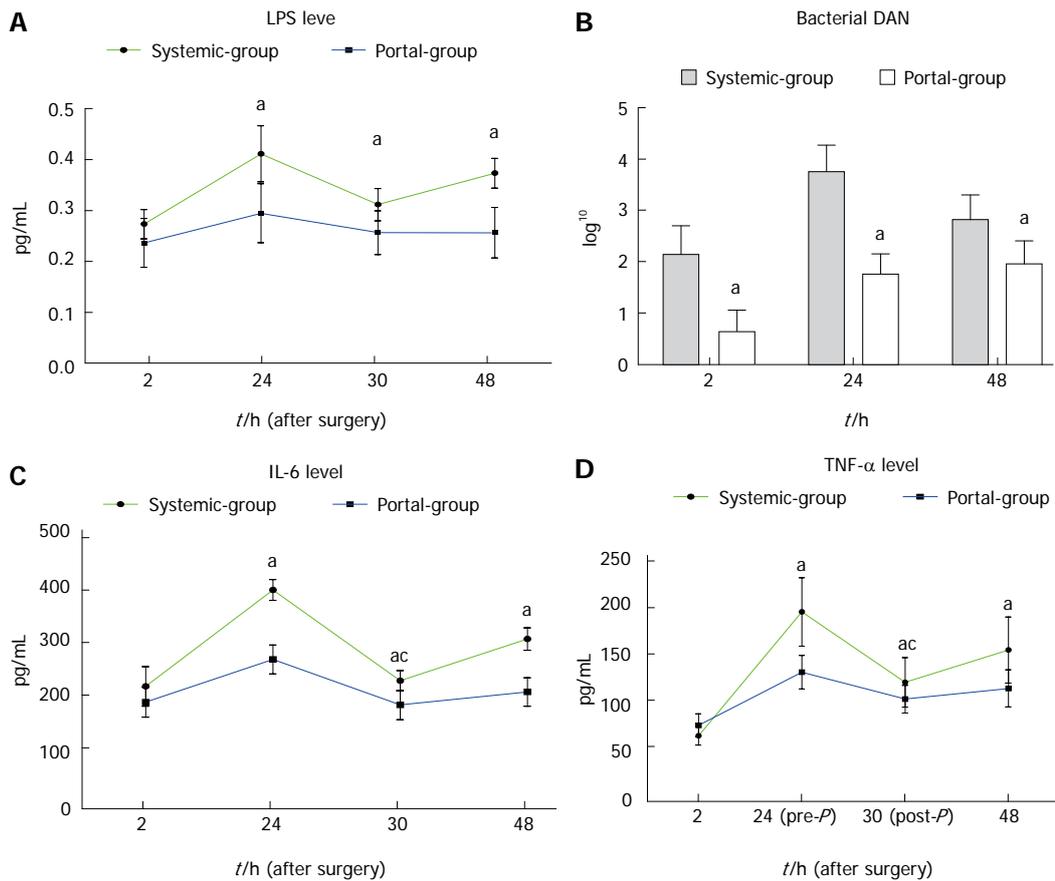
**Rate of liver remnant regeneration was elevated while apoptosis was attenuated in the Portal group compared with the Systemic group**

The rate of increase of liver volume in the Portal group was slightly increased compared with the Systemic group, although there was no statistical difference (*P* > 0.05)

(Figure 4A). Thymidine kinase activity was initially lower in the Portal group than the Systemic group immediately PH, and was subsequently higher at 48 h PH (Figure 4D). The PCNA index (PI) was significantly increased and the apoptotic index (AI) was significantly decreased in the Portal group compared with the Systemic group (Figure 4C, F). These results suggested that the rate of liver remnant regeneration was elevated, while the rate of apoptosis was attenuated in the Portal group compared with the Systemic group.

**Bacterial translocation and endotoxin as well as the inflammatory response were significantly attenuated in the Portal group compared with the Systemic group**

Serial changes in serum LPS levels, bacterial DNA levels, TNF-α and IL-6 levels in the Portal group compared



**Figure 5** Bacterial translocation and endotoxin, as well as the inflammatory response, were significantly attenuated in the Portal group compared with the Systemic group. A: Lipopolysaccharide (LPS) level was reduced in the Portal group compared with the Systemic group from 24 h post-hepatectomy (PH) until 48 h PH; B: Bacterial DNA level was reduced in the Portal group compared with the Systemic group from 2 h PH until 48 h PH; C: Interleukin (IL)-6 level was reduced in the Portal group compared with the Systemic group from 24 h PH until 48 h PH. D: tumor necrosis factor (TNF)-α level was reduced in the Portal group compared with the Systemic group from 24 h PH until 48 h PH. <sup>a</sup>*P* < 0.05 indicates Portal group vs Systemic group; <sup>c</sup>*P* < 0.05 indicates before plasma exchange vs after plasma

with the Systemic group are shown in Figure 5. The results showed that LPS, TNF-α and IL-6 levels were all significantly decreased in the Portal group compared with the Systemic group from 24 to 48 h PH, while bacterial DNA level was decreased from 2 to 48 h PH (Figure 5). In addition, LPS, bacterial DNA, TNF-α and IL-6 levels were all significantly decreased after plasma exchange from 24 to 30 h PH. These results suggested that bacterial translocation and endotoxin as well as the inflammatory response were significantly attenuated in the Portal group compared with the Systemic group.

## DISCUSSION

Following extensive hepatectomy when the remnant liver mass is low, it is unable to sustain synthetic, metabolic and detoxifying functions, and SFSS or PLF may ensue<sup>[5,6]</sup>. SFSS is a recognizable clinical syndrome, which is characterized by postoperative liver dysfunction with prolonged cholestasis, coagulopathy and portal hypertension. The mortality in severe SFSS or PLF after hepatectomy and LDLT is very high and ranges from 80% to 100%<sup>[5,20]</sup>.

PP or plasma exchange, a type of plasma purification, is usually performed in acute liver failure. During this process, the patient’s blood is introduced into the plasma

separator and the plasma is replaced. The harmful substances or protein-binding toxins are eliminated from the blood, and the blood cells and fresh frozen plasma are re-infused. PP can eliminate toxic soluble materials and small-molecule toxins, as well as coagulation factors, opsonins, and albumin among other factors<sup>[21]</sup>. In previous research<sup>[1,2,22,23]</sup>, it was demonstrated that these modalities can temporarily support the metabolic and excretory functions of the liver, and help to remove potentially hepatotoxic substances and maintain the patient’s clinical stability. The present study demonstrated that PP *via* both the systemic circulation and the portal vein can reduce toxic load and the inflammatory response and improve liver function, blood coagulation status, and LPS translocation. However, we were unable to find literature reports on the successful treatment of PLF<sup>[24-26]</sup>.

In SFSS or PLF following massive hepatectomy or LDLT, the toxic load is not “solely” pathogenic, as portal hypertension and splanchnic pooling have been reported to greatly contribute to the high postoperative morbidity and mortality of SFSS or PLF<sup>[6,24]</sup>. Severe damage to the sinusoidal endothelial cells (SECs) of the remnant liver is one of the main factors responsible for high mortality<sup>[5,16,27]</sup>. Sinusoidal overperfusion seems to be a significant factor impairing liver function following

liver resection. PP *via* systemic circulation access, which is currently universally adopted, did not improve survival rate and relieve portal hyperperfusion<sup>[2,25,28]</sup>. In the present study, the Portal group undergoing ECPD plus PP *via* the portal vein not only demonstrated removal of the toxic load, but also diversion of portal flow to the systemic circulation, thus relieving portal hypertension. This method attenuated sinusoidal endothelial injury and hepatocyte injury, and significantly decreased the serum endotoxin/bacterial DNA level, IL-6, and TNF- $\alpha$  level compared with the Systemic group without portal decompression. These results also indicated that portal hypertension not only damages the sinusoidal endothelium, but also aggravates endotoxin absorption/bacterial translocation<sup>[7,27,29]</sup>. Therefore, ECPD plus PP *via* the portal vein, which relieved both toxic load and portal hyperperfusion injury, has an advantage over PP *via* the systemic circulation. In a recent report, it was identified that PP combined with surgical modulation of the portal vein inflow was an effective treatment for SFSS after LDLT<sup>[26]</sup>.

Currently, the portacaval or mesocaval shunt is usually adopted to relieve portal hyperperfusion in both the clinic and in animal experiments. However, these techniques have many shortcomings, including surgical procedure-related complications and, long-lasting and excessive diversion of portal flow which could retard liver regeneration<sup>[16,30,31]</sup>. Fortunately, dynamic adjustment of the diverting flow between the portal and systemic circulation is characteristic of ECPD, which was able to halt the portal diversion, while the liver remnant underwent hypertrophy. This showed a potential advantage over the modalities presently adopted. In this study, the PVF per unit volume in the Portal group was preserved at more than 3 times the baseline value, and the increased rate of the liver remnant in the Portal group at 48 h PH was similar to that in the Systemic group with the presence of higher portal hyperperfusion. This indicated that PVF preserved at more than 3 times the baseline value was adequate and a good stimulus for liver regeneration. In addition, injury to the liver in the Portal group was milder and the AI was significantly lower than that in the Systemic group. To the best of our knowledge, this is the first study to investigate the feasibility and effectiveness of ECPD plus PP in relieving portal hyperperfusion in PLF or SFSS. As residual liver increases rapidly after major hepatectomy and within two days, portal hypertension will be relieved rapidly. Thus, ECPD is usually only needed for a short time. In this study, even when ECPD was stopped, the PVP only rose slightly compared with the baseline value, indicating that portal hypertension was relieved after a short time.

In general, ECPD plus temporal PP or plasma purification *via* the portal vein does not only dynamically turn the portal flow to the systemic circulation, attenuate portal overflow injury, and preserve the optimum portal flow for liver regeneration, but also reduces toxic load and improves biochemistry parameters. This technique should be undertaken instead of PP or ALS *via* the systemic circulation in SFSS or PLF.

## COMMENTS

### Background

Plasmapheresis (PP) and other plasma purification modalities have been used in the past to treat postoperative liver failure (PLF) and “small-for-size” syndrome (SFSS). However, these modalities have not resulted in a significant improvement in survival. It is thought that these modalities were unable to relieve portal hypertension, thus are inefficacious.

### Research frontiers

In SFSS or PLF after hepatectomy and living donor liver transplantation, the rationale for using PP or other plasma purification modalities is support for the patient, however, these modalities do not relieve portal hyperperfusion. The portacaval or mesocaval shunt (PCS/MCS) is usually adopted to relieve portal hyperperfusion, however, the long-lasting diversion of portal flow and the potential risk of excessive diversion of portal flow to the systemic circulation could retard liver regeneration.

### Innovations and breakthroughs

In SFSS and PLF, extracorporeal continuous portal diversion (ECPD) plus PP *via* the portal vein not only removes the toxic load, but continuously diverts the portal flow to the systemic circulation and relieves portal hypertension, attenuates sinusoidal endothelial injury and has an advantage over PP *via* the systemic circulation. In addition, ECPD *via* the portal vein can dynamically adjust the diverting flow to the “functional competition” between the portal and systemic circulation in the case of PCS/MCS, and is available to halt the portal diversion, while the remnant liver underwent hypertrophy. This showed a potential advantage of this technique over the modalities presently adopted.

### Applications

ECPD plus temporal PP *via* the portal vein does not only attenuate portal overflow injury, but also reduces toxic load and should be undertaken instead of PP or ALS *via* the systemic circulation in SFSS or PLF, and shows potential for application in the clinic.

### Peer review

It was supposed that PP and other artificial liver support (ALS) modalities did not relieve portal hypertension, resulting in inefficacious treatment. This study firstly demonstrated that the ECPD plus temporal PP *via* the portal vein can not only attenuate portal overflow injury, but also reduce toxic load, and should be undertaken instead of PP or ALS *via* the systemic circulation in SFSS or PLF.

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## Xiaotan Tongfu granules contribute to the prevention of stress ulcers

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### Abstract

**AIM:** To investigate the efficacy and potential mechanism of Xiaotan Tongfu granules (XTTF) in stress ulcers.

**METHODS:** One hundred sixty rats were randomly divided into 4 groups ( $n = 10$ ) as follows: the model group (MP group), the control group (CP group), the ranitidine group (RP group) and the XTTF granule group (XP group). Rats in the MP group received no drugs, rats in the CP group received 0.2 mL of a 0.9% sodium chloride solution *via* oral gavage, and rats in the RP and XP groups received the same volume of ranitidine (50 mg/kg) or XTTF granule (4.9 g/kg). The cold-restraint stress model was applied to induce stress ulcers after 7 consecutive days of drug administration. Afterwards, rats were sacrificed at 0, 3, 6 and 24 h. Gastric pH was measured by a precise pH meter;

gastric emptying rate (GER) was measured by using a methylcellulose test meal; myeloperoxidase activity (MPO), macrophage migration inhibitory factor (MIF), proliferating cell nuclear antigen (PCNA), and heat shock protein 70 (HSP70) were measured by immunohistochemical staining; and mucosal cell apoptosis was measured by transferase dUTP nick end labeling.

**RESULTS:** In the cold-restraint stress model, the development of stress ulcers peaked at 3 h and basically regressed after 24 h. Gastric lesions were significantly different in the RP and XP groups at each time point. Interestingly, although this index was much lower in the RP group than in the XP group immediately following stress induction (7.00  $\pm$  1.10 *vs* 10.00  $\pm$  1.79,  $P < 0.05$ ). Concerning gastric pH, between the RP and XP groups, we detected a statistically significant difference immediately after stress induction (0 h: 4.56  $\pm$  0.47 *vs* 3.34  $\pm$  0.28,  $P < 0.05$ ) but not at any of the subsequent time points. For GER, compared to the RP group, GER was remarkably elevated in the XP group because a statistically significant difference was detected (3 h: 46.84  $\pm$  2.70 *vs* 61.16  $\pm$  5.12,  $P < 0.05$ ; 6 h: 60.96  $\pm$  6.71 *vs* 73.41  $\pm$  6.16,  $P < 0.05$ ; 24 h: 77.47  $\pm$  3.17 *vs* 91.31  $\pm$  4.34,  $P < 0.05$ ). With respect to MPO and MIF, comparisons between the RP and XP groups revealed statistically significant differences at 3 h (MPO: 18.94  $\pm$  1.20 *vs* 13.51  $\pm$  0.89,  $P < 0.05$ ; MIF: 150.67  $\pm$  9.85 *vs* 122.17  $\pm$  5.67,  $P < 0.05$ ) and 6 h (MPO: 13.22  $\pm$  1.54 *vs* 8.83  $\pm$  0.65,  $P < 0.05$ ; MIF: 135.50  $\pm$  9.46 *vs* 109.83  $\pm$  6.40,  $P < 0.05$ ). With regard to HSP70, HSP70 expression was significantly increased in the RP and XP groups at 3 and 6 h compared to the MP and CP groups. In addition, comparing the RP and XP groups also showed statistically significant differences at 3 and 6 h. The expression of PCNA was higher in the RP and XP groups 3 h after stress induction. Between these two groups, small but statistically significant differences were observed at all of the time points (3 h: 69.50  $\pm$  21.52 *vs* 79.33  $\pm$  15.68,  $P < 0.05$ ;

6 h:  $107.83 \pm 4.40$  vs  $121.33 \pm 5.71$ ,  $P < 0.05$ ; 24 h:  $125.33 \pm 5.65$  vs  $128.50 \pm 14.49$ ,  $P < 0.05$ ) except 0 h. With regard to apoptosis, the apoptotic activity in the RP and XP groups was significantly different from that in the MP and CP groups. The XP group exhibited a higher inhibition of cell apoptosis than the RP group at 3 h ( $232.58 \pm 24.51$  vs  $174.46 \pm 10.35$ ,  $P < 0.05$ ) and 6 h ( $164.74 \pm 18.31$  vs  $117.71 \pm 12.08$ ,  $P < 0.05$ ).

**CONCLUSION:** The Xiaotan Tongfu granule was demonstrated to be similar to ranitidine in preventing stress ulcers. It exhibited multiple underlying mechanisms and deserves further study.

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**Key words:** Stress ulcer; Xiaotan Tongfu granule; Inflammation; Heat shock protein 70; Proliferation and apoptosis; Gastric emptying rate

**Core tip:** Although the underlying mechanism of stress ulcers is commonly believed to depend on the balance between known aggressive factors and mucosal defense mechanisms, most clinical strategies still aim to inhibit gastric acid. In this study, we demonstrated that the Xiaotan Tongfu granule was similar to ranitidine treatment in reducing gastric lesions in a cold-restraint stress model. The underlying mechanisms may include acceleration of the gastric emptying rate, inhibition of local inflammation, promotion of cell proliferation and suppression of apoptosis. Our study indicated that multiple manipulations of the factors involved in inducing stress ulcers could be as effective as simple acid inhibition.

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## INTRODUCTION

Stress ulceration (SU) has been conventionally regarded as an inevitable complication of the gastrointestinal tract in people experiencing abnormally high physiological stress (*e.g.*, trauma, surgery, organ failure, sepsis, or burn)<sup>[1]</sup>. Gastrointestinal bleeding is a life-threatening complication of SU and was observed in 64% of patients with SU, compared to only 9% of patients without SU, in a previous study<sup>[2]</sup>. It is believed that clinically significant gastrointestinal bleeding in critically ill patients is associated with increased mortality rates, lengthened intensive care unit stays and additional costs<sup>[3-5]</sup>.

The development of stress ulcers is largely determined by the balance between known aggressive factors and defense mechanisms. The former usually include

gastric acid<sup>[6]</sup>, abnormal motility<sup>[7]</sup>, and *Helicobacter pylori* infection<sup>[8,9]</sup>, and the latter include heat shock protein<sup>[10]</sup>, cellular regeneration<sup>[11]</sup>, *etc.* SU prophylaxis (SUP) was thought to play a pivotal role in the care of critically ill patients, and it was reported that appropriate SUP could decrease mortality. At present, although multiple protocols are available for SUP, there are no universally accepted regimens<sup>[12]</sup>. Nevertheless, the evidence that the appropriate application of some pharmacologic agents, such as proton pump inhibitors, histamine-2 receptor antagonists, and sucralfate<sup>[13]</sup>, could decrease the risk of bleeding has been long established.

Traditional Chinese medicine (TCM) has been demonstrated to be effective in the management of stress-related gastrointestinal disorders, including irritable bowel syndrome<sup>[14,15]</sup>, and a number of studies have also indicated that TCM could exert measurable therapeutic effects on gastric ulcers in rats<sup>[16-18]</sup>. Based on these studies on TCM, the Xiaotan Tongfu (XTTF) granule (Table 1), which is primarily composed of a Xiao-cheng-qi decoction<sup>[19]</sup> and a Xiao-ban-xia decoction<sup>[20]</sup> (two ancient herbal formulas originating from the Treaty of Febrile and Miscellaneous written by Zhongjing Zhang in the years of 25-220 AD during the Eastern Han Dynasty), was used to treat gastrointestinal disorders in critically ill patients at our hospital. The rationale behind this treatment was that previous studies have indicated that the granule could improve the Acute Physical and Chronic Health Evaluation scores in patients experiencing gastrointestinal dysfunction (unpublished data). Considering this background, we speculated that the XTTF granule could be applied to the management of SU. In the present study, we investigated the efficacy of the XTTF granule in SU and the potential mechanisms involved.

## MATERIALS AND METHODS

### Animals

One hundred sixty male Sprague-Dawley rats weighing 200-220 g were purchased from Xipuier-Bikai Experimental Animal Co. LTD (Shanghai). The animals were housed in cages with wide mesh wire bottoms to prevent coprophagy, fed a standard laboratory diet and given free access to tap water. The cages were kept in a room with controlled temperature ( $22 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ ), relative humidity (65%-70%) and day/night cycle (12:12 light/dark). All of the rats were handled according to the recommendations of the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. The protocol was approved by the Shanghai Medical Experimental Animal Care Commission.

### Drug administration

The XTTF granule was manufactured by Tian Jiang Pharmacy Co. Ltd (Jiangyin, China) and supervised by the Changzheng Hospital of the Second Military Medical University with the assigned batch number 1011370. We established the granule under the guidance of TCM

**Table 1** Ingredients and the corresponding percent of Xiaotan Tongfu granules

Chinese name	Common name	Latin name	Percent
Da Huang	Rhubarb	<i>Rhei Radix Et Rhizoma</i>	10%
Zhi Shi	Immature Bitter Orange	<i>Aurantii Fructus Immaturus</i>	10%
Ban Xia	Pinellia Tuber	<i>Pinelliae Rhizoma</i>	10%
Hou Pu	Magnolia Bark	<i>Magnoliae Officinalis Cortex</i>	6%
Bai Shao	White Peony Root	<i>Radix Paeoniae Alba</i>	10%
Xi Xin	Manchurian Wild Ginger	<i>Asari Radix Et Rhizoma</i>	4%
Huang Lian	Coptis Root	<i>Coptidis Rhizoma</i>	4%
Pu-Gong Yin	Dandelion	<i>Asari Radix Et Rhizoma</i>	10%
Bai-Hua-She-She Cao	Snake-needle Grass	<i>Hedyotis Diffusa</i>	10%
Fo Shou	Finger Citron	<i>Citri Sarcodactylis Fructus</i>	10%
Xiang Yuan	Citron Fruit	<i>Citri Fructus</i>	10%
Gan Cao	Licorice Root	<i>Glycyrrhizae Radix Et Rhizoma</i>	6%

related to stress ulcers<sup>[21]</sup>, and some of the components were previously shown to be effective in the management of stress-related symptoms. For example, the Xiao-ban-xia decoction could elevate gastric emptying<sup>[22]</sup>, which was delayed under stress conditions. In addition, the major component of the Xiao-cheng-qi decoction, *Rhei Radix Et Rhizoma*, was demonstrated to be effective in the prevention of stress ulcers *via* multiple mechanisms<sup>[23,24]</sup>. The rats were randomly divided into 4 groups ( $n = 10$ ) as follows: the model group (MP group), the control group (CP group), the ranitidine group (RP group) and the XTTF granule group (XP group). Rats in the MP group received no drugs; rats in the CP group received 0.2 mL of a 0.9% sodium chloride solution *via* oral gavage; and rats in the RP and XP groups received the same volume of ranitidine (50 mg/kg)<sup>[25-27]</sup> or XTTF granule (4.9 g/kg, corresponding to twice that of an adult human dose), respectively. The administration frequency was twice daily and sustained for 7 d. On the 8<sup>th</sup> day, rats were starved for 24 h (free of water) and prepared for the stress experiment.

### Induction of stress ulceration

The cold-restraint stress model used in the present study was originally devised by Senay *et al.*<sup>[28]</sup> and modified by Wong *et al.*<sup>[29]</sup>. Briefly, rats were restrained inside individual close-fitting tubular wire mesh cages and exposed to an ambient temperature of 4 °C for 3 h. Rats were anesthetized and sacrificed at 0, 3, 6 and 24 h after stress induction, and the stomachs were opened along the greater curvature. After measuring the mucosal lesions, sections of the tissues were fixed in 10% buffered formalin solution and stained for proliferating cell nuclear antigen (PCNA), heat shock protein 70 (HSP70), and macrophage migration inhibitory factor (MIF) *via* immunohistochemistry (IHC) and for apoptosis *via* transferase dUTP nick end labeling (TUNEL) staining.

### Measurement of gastric ulcer index, pH and emptying rate

The severity of the mucosal lesions was determined using a magnifier ( $\times 10$ ) and rated for gross pathology according to the scale of ulcer scores as described by Dekanski *et al.*<sup>[30]</sup> with a modification introduced by Martín *et al.*<sup>[31]</sup>. For every group, 4 rats were used in the precise measurement of gastric pH, and the test was performed by 3 independent investigators to determine the mean pH. Gastric emptying ( $n = 6$ , 2 rats were used as a control in each group) was measured using a methylcellulose test meal, as previously described<sup>[32,33]</sup>.

### Measurement of myeloperoxidase activity in the gastric mucosa

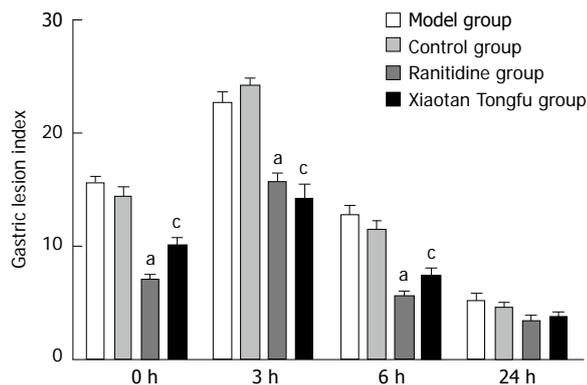
Myeloperoxidase (MPO) activity was determined by the method described by Bradley *et al.*<sup>[34]</sup> with some modifications<sup>[35]</sup>. The gastric mucosa was homogenized in a potassium phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide, and the supernatant was assayed for MPO activity. The sample was mixed with hydrogen peroxide and *O*-Dianisidine prepared in a potassium phosphate buffer solution. The end point absorbance of the mixture was measured at 460 nm using a spectrophotometer with horseradish peroxidase as a standard. The protein assay was conducted using the method described by Lowry *et al.*<sup>[36]</sup>.

### Immunohistochemical staining for PCNA, HSP70 and MIF

Tissues were fixed in 10% formalin, embedded in paraffin, and processed by standard histological methods. From each paraffin block, 5- $\mu$ m serial sections were sliced. IHC studies were performed with kits utilizing the avidin-biotin-peroxidase complex according to the manufacturer's instructions (Invitrogen, United States). Primary antibodies [anti-PCNA (rabbit polyclonal, dilution 1:50, BD Biosciences) anti-HSP70 (rabbit polyclonal, dilution 1:50, BD Biosciences, United States), and anti-MIF (rabbit polyclonal, dilution 1:100, BD Biosciences, United States)] were incubated at room temperature overnight in a humidified chamber. The positive results were stained brown and counted by the Image Pro Express system (Olympus, Japan) at  $\times 400$  magnification (BX51, Olympus, Japan); the method of calculation was introduced by Soslow *et al.*<sup>[37]</sup>.

### Measurement of apoptotic cells in the gastric mucosa

Apoptosis measurement was detected by TUNEL staining according to the method of Gavrieli *et al.*<sup>[38]</sup>. After digestion with proteinase K, the tissues were treated with H<sub>2</sub>O<sub>2</sub> solution and washed with distilled water. The sections were then covered with TdT buffer containing TdT and biotinylated dUTP. The reaction was halted by washing the sections with a 3% H<sub>2</sub>O<sub>2</sub> methanol solution at room temperature. After blocking the non-specific binding with normal diluted serum, sections were incubated with peroxidase-labeled streptavidin and stained with



**Figure 1** Results of the gastric lesion index ( $n = 6$  for each group). At 0 h, ranitidine was demonstrated to be the most powerful agent in the inhibition of gastric lesions. The difference in the inhibition of gastric lesions between the ranitidine group and the Xiaotan Tongfu granule group was statistically significant ( $P < 0.05$ ). However, at the subsequent time points, this difference vanished. <sup>a</sup> $P < 0.05$  vs the model group; <sup>c</sup> $P < 0.05$  vs the control group.

diaminobenzidine-H<sub>2</sub>O<sub>2</sub>. Finally, the sections were counterstained with Mayer’s hematoxylin. Sections treated with DNase I in buffer solution served as the positive control, whereas the negative control was prepared by omitting the TdT from the buffer solution. The positive cells were counted by the Image Pro Express system (Olympus) at  $\times 400$  magnification (BX51, Olympus). The apoptotic index was defined as the average number from 10 to 25 glands of each mucosal section.

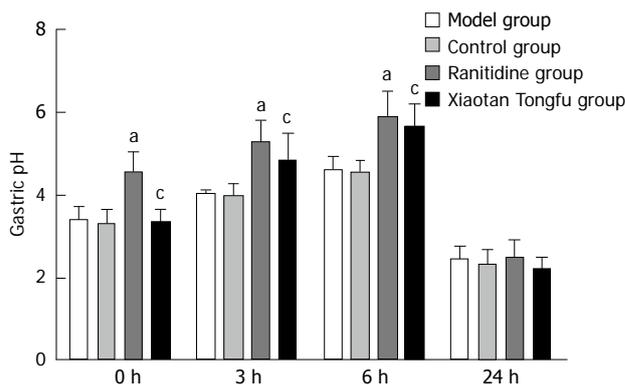
**Statistical analysis**

All data were processed by SPSS 18.0 and presented as the mean  $\pm$  SD. Comparisons between the different groups were evaluated by a one-way analysis of variance followed by the Bonferroni test. Values of  $P < 0.05$  were considered to be statistically significant. To avoid subjective bias of the parameters measured in this study, observers were blinded to the sample sources at the time of assessment.

**RESULTS**

**XTTF granule shows similar capabilities as ranitidine in reducing gastric lesions**

As shown in Figure 1, gastric lesions developed in a time-dependent manner and peaked at 3 h after stress induction; at 24 h after stress induction, these lesions had regressed. In the MP and CP groups, no statistically significant differences in this index were detected either overall or at each of the individual time points ( $P > 0.05$ ). In the RP and XP groups, gastric lesions were significantly different compared to the MP and CP groups at each time point ( $P < 0.05$ ), except at 24 h after stress induction ( $P > 0.05$ ). Interestingly, although this index was much lower in the RP group than in the XP group immediately after the stress ( $7.00 \pm 1.10$  vs  $10.00 \pm 1.79$ , respectively;  $P < 0.05$ ), this difference was eliminated at 3 h ( $15.67 \pm 1.97$  vs  $14.17 \pm 3.125$ , respectively;  $P > 0.05$ ), 6 h ( $5.50 \pm 1.05$  vs  $7.33 \pm 1.63$ , respectively;  $P > 0.05$ ) and 24 h ( $1.67$



**Figure 2** Results of gastric pH ( $n = 4$  in each group). At 0 h, ranitidine was demonstrated to be the most powerful agent in increasing the gastric pH, and the increase in the gastric pH was significantly different between the ranitidine group (RP group) and the Xiaotan Tongfu granule group (XP group) ( $P < 0.05$ ). In addition, there were no significant differences regarding the increase in the gastric pH among the XP, the model group (MP group), the control group (CP group) ( $P > 0.05$ ). At 3 h and 6 h after stress induction, there were no differences in the gastric pH between the RP and XP groups. <sup>a</sup> $P < 0.05$  vs the MP group; <sup>c</sup> $P < 0.05$  vs the CP group.

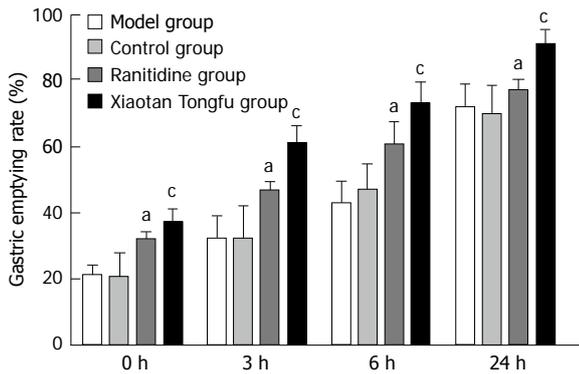
$\pm 0.52$  vs  $1.50 \pm 0.55$ , respectively;  $P > 0.05$ ) after stress induction.

**Gastric pH in the XP and RP groups is significantly lower than in the MP and CP groups**

As shown in Figure 2, the fluctuation of gastric pH was restricted to a limited range, except for 24 h after stress induction. There were no significant differences observed between the MP and CP groups ( $P > 0.05$ ). In the RP and XP groups, we detected a statistically significant difference immediately after stress induction ( $4.56 \pm 0.47$  vs  $3.34 \pm 0.28$ , respectively;  $P < 0.05$ ) but not at any of the subsequent time points ( $P > 0.05$ ). The gastric pH also recovered to normal levels 24 h after stress induction in these two groups, and no significant differences were observed among all of the groups ( $P > 0.05$ ).

**XTTF granule and ranitidine treatment accelerated the gastric emptying rate**

It has been established that stress could produce a marked delay of gastric emptying in both humans and animals<sup>[39,40]</sup>. As shown in Figure 3, the gastric emptying rate (GER) was remarkably suppressed very shortly after stress induction and was gradually restored over time. This effect was obvious in the MP and CP groups, and no significant differences were observed between these groups ( $P > 0.05$ ). Previous studies had shown that ranitidine could accelerate the GER in stress conditions<sup>[41,42]</sup>, and our study echoed this conclusion. In addition, we were intrigued by the greater improvement in GER for the XP group because a statistically significant difference was detected immediately after stress induction ( $46.84 \pm 2.70$  vs  $61.16 \pm 5.12$ , respectively;  $P < 0.05$ ), at 3 h ( $60.96 \pm 6.71$  vs  $73.41 \pm 6.16$ , respectively;  $P < 0.05$ ) and at 6 h ( $77.47 \pm 3.17$  vs  $91.31 \pm 4.34$ , respectively;  $P < 0.05$ ) compared to the RP group. This difference was sustained



**Figure 3** Results of the gastric emptying rate ( $n = 6$ , 2 rats were used as controls for each group). At 0 h, the Xiaotan Tongfu granule group (XP group) was superior in elevating the gastric emptying rate (GER); however, no significant difference was detected between the ranitidine group (RP group) and the XP group at this point ( $P > 0.05$ ). At 3, 6 and 24 h after stress induction, the GER in the XP group was sustained at a high value and was significantly different compared to the RP group ( $P < 0.05$ ). <sup>a</sup> $P < 0.05$  vs the model group; <sup>c</sup> $P < 0.05$  vs the control group.

at 24 h after stress induction ( $P < 0.05$  compared to all of the groups).

#### **XTTF granule and ranitidine inhibited local inflammation**

Tissue MPO levels were correlated with the neutrophil levels and served as a marker of neutrophil infiltration<sup>[43]</sup>. MIF, a 12.5-kDa cytokine, has increasingly been recognized for its proinflammatory properties in the inflammatory process in SU<sup>[43,44]</sup>. In our study, as shown in Figure 4, the variation of local inflammation (MPO and MIF) resembled the gastric pH. No significant differences were observed between the MP and CP groups, but comparisons between the RP and XP groups revealed statistically significant differences at 3 h ( $18.94 \pm 1.20$  vs  $13.51 \pm 0.89$ , respectively;  $P < 0.05$ ) and 6 h ( $13.22 \pm 1.54$  vs  $8.83 \pm 0.65$ , respectively;  $P < 0.05$ ) after stress induction.

#### **XTTF granule and ranitidine promoted the expression of HSP70**

Numerous studies have suggested that HSP70 could provide protection against gastric ulcers *via* multiple mechanisms<sup>[45]</sup>. As shown in Figure 5, there was a measurable expression of HSP70 3 h after stress induction, and this expression peaked at 6 h. No significant differences regarding HSP70 expression were observed between the MP and CP groups at any of the time points, but HSP70 expression was significantly higher in the RP and XP groups at 3 and 6 h compared to the MP and CP groups ( $P < 0.05$ ). In addition, comparison of the RP and XP groups also yielded statistically significant differences at 3 h ( $133.33 \pm 35.53$  vs  $176.17 \pm 9.37$ , respectively;  $P < 0.05$ ) and 6 h ( $182.83 \pm 38.78$  vs  $226.50 \pm 18.84$ , respectively;  $P < 0.05$ ) after stress induction.

#### **XTTF granule and ranitidine promote cell proliferation and inhibit gastric mucosal cell apoptosis**

As shown in Figure 6, cell proliferation varied in a time-

dependent manner and increased gradually after stress induction. No significant differences were observed between the MP and CP groups ( $P > 0.05$ ). In contrast with these groups, the expression of PCNA was higher in the RP and XP groups 3 h after stress induction ( $P < 0.05$ ), with small but significant differences observed at all of the time points except 0 h ( $37.50 \pm 10.91$  vs  $40.83 \pm 1.56$ , respectively;  $P > 0.05$ ) between these two groups. Peak apoptotic activity was observed at 3 h and returned to normal levels over time, as shown in Figure 6. There were no significant differences regarding apoptotic cells between the MP and CP groups, but the apoptotic activity in the RP and XP groups was significantly different from that in the MP and CP groups ( $P < 0.05$ ). Treatment in the XP group led to a higher inhibition of cell apoptosis than in the RP group at 3 h ( $232.58 \pm 24.51$  vs  $174.46 \pm 10.35$ , respectively;  $P < 0.05$ ) and 6 h ( $164.74 \pm 18.31$  vs  $117.71 \pm 12.08$ , respectively;  $P < 0.05$ ), but 24 h after stress induction, no significant differences could be detected between either of the groups.

## **DISCUSSION**

In the present study, the antiulcer effect of the Xiaotan Tongfu granule was established, and its efficacy was demonstrated to be similar to that of ranitidine. The cold-restraint stress model induced a series of pathological alterations and lesions in the stomach, which, when examined together with previous studies, suggested that SU is a process that results from multiple sources<sup>[46,47]</sup>. We concluded that although the XTTF granule was inferior to ranitidine in reducing gastric acid secretion immediately after stress induction, this did not impair its efficacy because the XTTF granule was superior in promoting a series of parameters, including inhibited local inflammation, increased GER, enhanced HSP70 expression, decreased cell apoptosis and elevated cell proliferation over time. The majority of these parameters have been demonstrated to contribute to ulcer prevention and healing<sup>[45]</sup>, which was confirmed by our observations of gastric lesions measured at the designated time points. Based on these results, we speculate that any agents that can interfere with the above parameters either individually or collectively would be useful to ameliorate any complications due to stomach ulcers.

The underlying mechanism of SU was previously not thoroughly understood and was commonly believed to depend on the balance between known aggressive factors and mucosal defense mechanisms<sup>[47]</sup>. Previous studies indicated that some components in our decoction, for example, the *Magnoliae Officinalis Cortex*, *Coptidis Rhizoma* and *Glycyrrhizae Radix Et Rhizoma*, were effective in inhibiting gastric acid secretion by a potential mechanism of regulating the activity of various postsynaptic gastric receptors such as histamine H2<sup>[48,49]</sup>. It was interesting that the XTTF granule was less efficacious in reducing gastric acid secretion immediately after stress induction and resulted in more serious gastric lesions compared to ra-

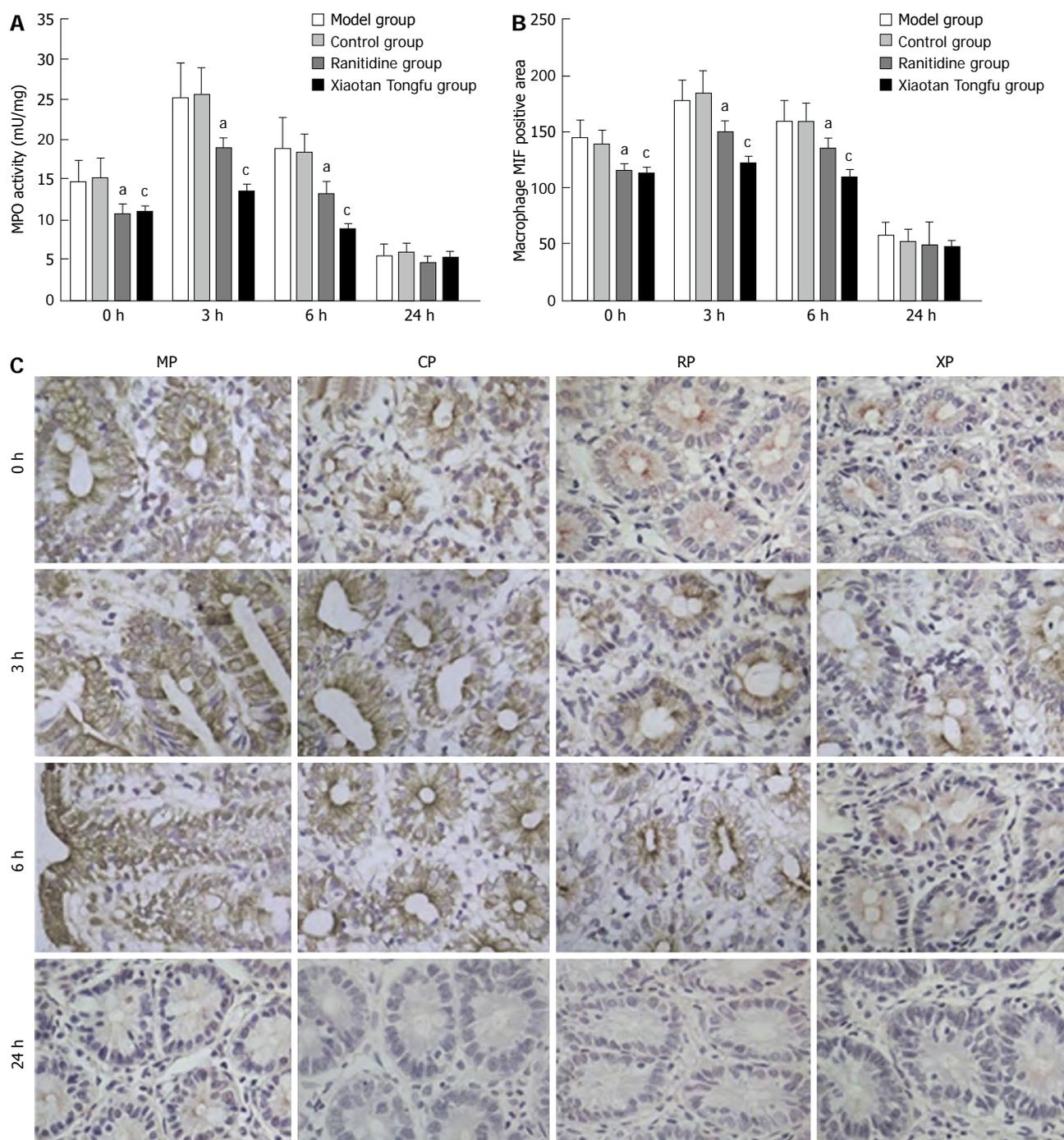
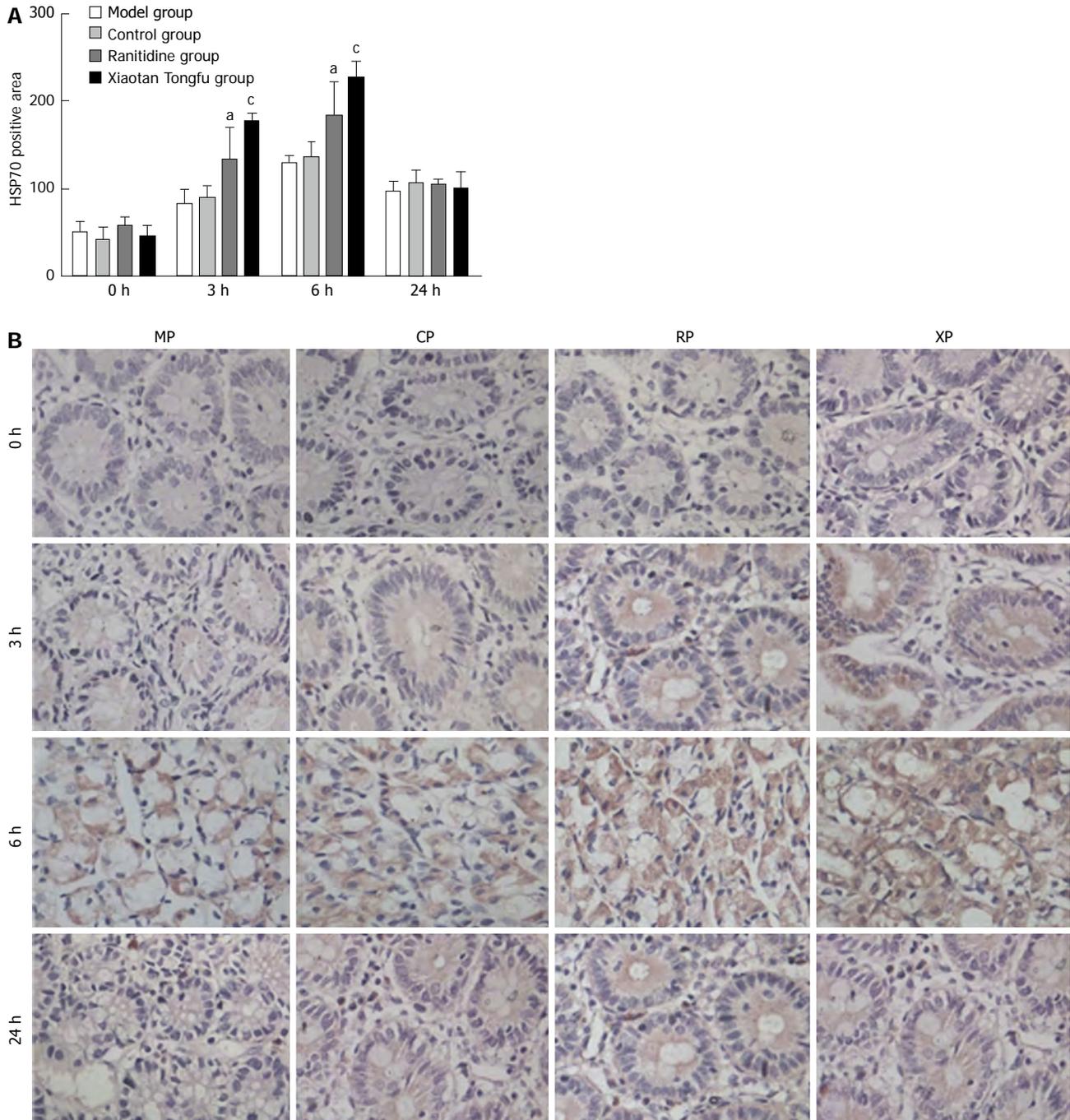


Figure 4 Measurement of myeloperoxidase activity and macrophage migration inhibitory factor ( $n = 6$  for each group). A, B: The variation of myeloperoxidase activity (MPO) activity and migration inhibitory factor (MIF) was similar. At 0 h, no significant difference was detected between the ranitidine group (RP group) and the Xiaotan Tongfu granule group (XP group) ( $P > 0.05$ ). However, 3 and 6 h after stress induction, these two indexes were inhibited in the XP group, which was a statistically significant difference compared to the RP group ( $P < 0.05$ ). <sup>a</sup> $P < 0.05$  vs the model group (MP group); <sup>c</sup> $P < 0.05$  vs the control group (CP group); C: The immunohistochemical staining results of MIF show that it was expressed in the cytoplasm of gastric epithelial cells and lamina propria cells. Original magnification  $\times 400$ .

nitidine. These results could be regarded as a footnote in that gastric acid is one of the most important factors in the formation of SU<sup>[50]</sup>. However, it should also be noted that not all clinically observed gastrointestinal bleeding can be prevented by manipulating the gastric pH<sup>[51]</sup>. The XTTF granule was shown to significantly promote GER, echoing the results in our previous study (unpublished observations) that concluded that the XTTF granule

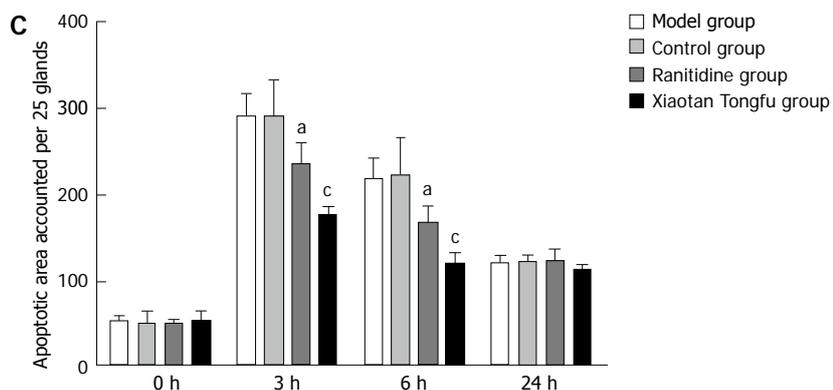
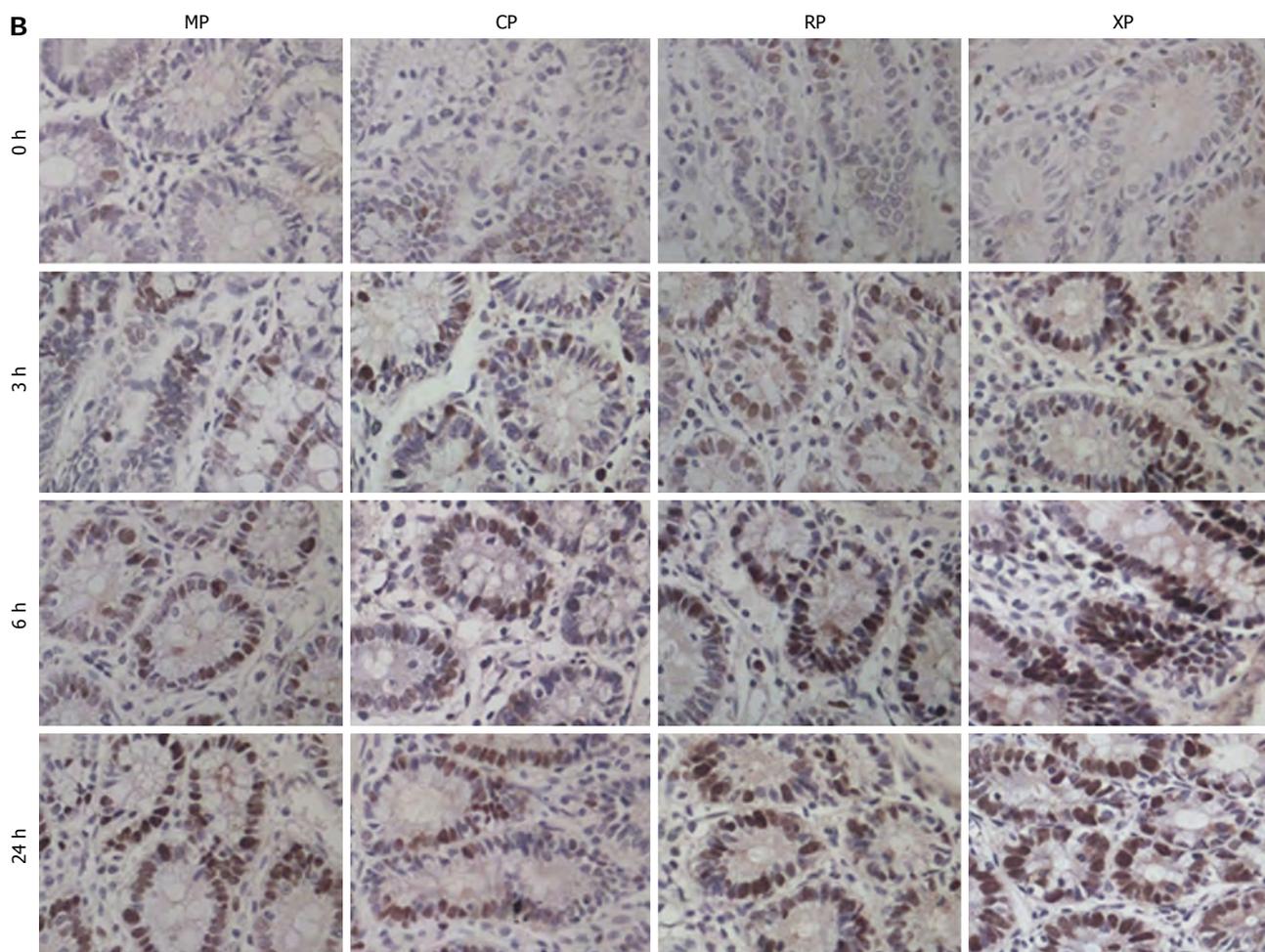
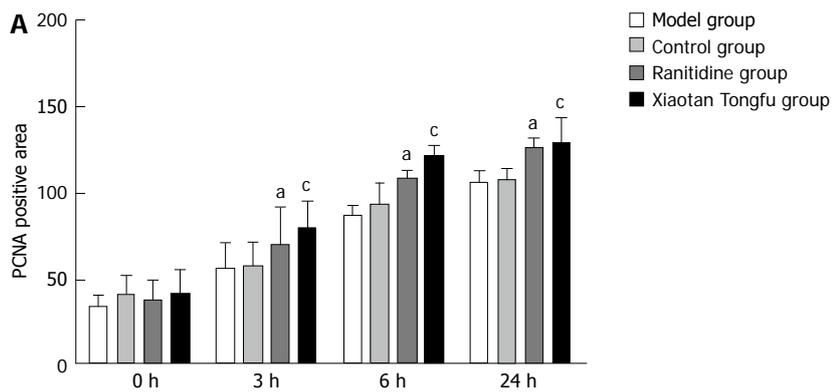
could enhance plasma motilin levels, which is important in gastric movement<sup>[52,53]</sup> in critically ill patients. Additionally, the Xiao-ban-xia decoction, which is an important component of the XTTF granule, has been demonstrated to be a regulative mediator of gastric motility<sup>[20]</sup>. An enhanced gastric emptying rate could remove acidic material and other irritants in the stomach<sup>[54]</sup>, which is beneficial for ulcer prevention. Additionally, it was notable that

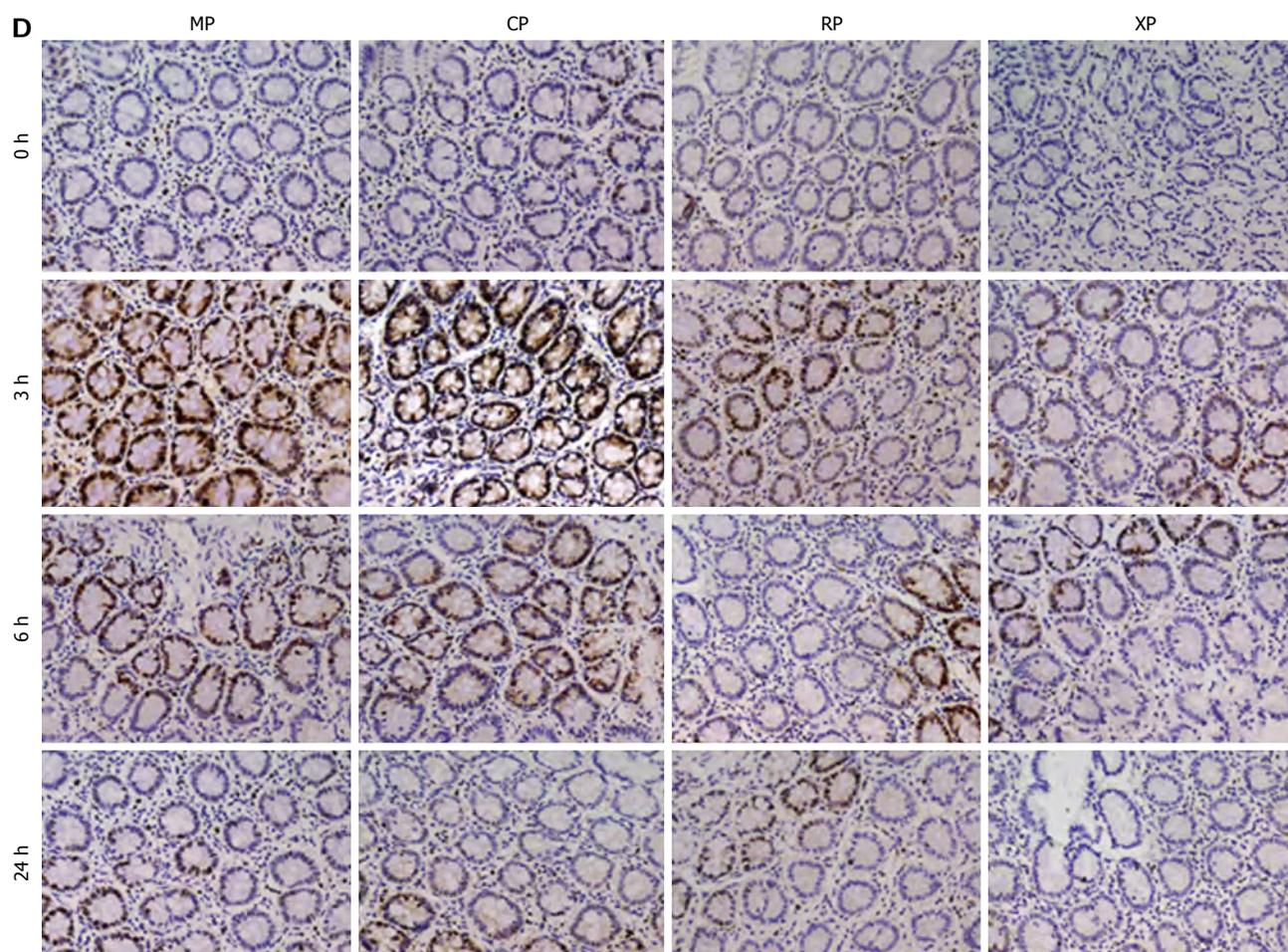


**Figure 5** Immunohistochemical staining results for heat shock protein 70 ( $n = 6$  for each group). **A:** There was a statistically significant difference in the protein expression levels between the ranitidine group (RP group) and the Xiaotan Tongfu granule group (XP group) groups at 3 and 6 h at the exact site of initial ulceration ( $P < 0.05$ ). <sup>a</sup> $P < 0.05$  vs the model group (MP group), <sup>c</sup> $P < 0.05$  vs the control group (CP group); **B:** Strong heat shock protein 70 (HSP70) immunoreactivity was observed in the gastric surface epithelium primarily in the nuclei, but protein was also observed in the cytoplasm. Original magnification  $\times 400$ .

some traditional Chinese herbal medicines were effective in preventing inflammation by various mechanisms, such as the inhibition of nuclear factor kappa B (NF- $\kappa$ B), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-17<sup>[55,56]</sup>. Interestingly, in our study, the XTTF granule was able to alleviate local inflammation by decreasing MPO activity and restraining MIF expression. It is well known that neutrophil adherence within the gastric microcirculation and migration into the gastric tissue are major causes of gastric ulcers<sup>[54]</sup>. MIF has been suggested to play a pivotal

role in this process, and anti-MIF treatment could have therapeutic value in SU<sup>[57]</sup>. Although the data indicating that the XTTF granule could inhibit MIF are limited, *Rhei Radix Et Rhizoma* (a main herb in the Xiao-cheng-qi decoction<sup>[19]</sup>) was previously shown to inhibit gastrointestinal inflammation by acting on TNF- $\alpha$ <sup>[24]</sup>, a strong inducer of MIF secretion<sup>[58]</sup>. Furthermore, XTTF granule can also improve local microcirculation<sup>[22]</sup>, thereby reducing neutrophil concentrations<sup>[59]</sup>. Other components, such as *Coptidis Rhizoma*, could ameliorate acute inflammation by





**Figure 6** Measurement of cell proliferation and mucosal cell apoptosis ( $n = 6$  for each group). A: The cell proliferation was significantly different between the ranitidine (RP) and Xiaotan Tongfu granule (XP) groups ( $P < 0.05$ ) at 3 and 6 h; B: Proliferating cell nuclear antigen (PCNA) immunoreactivity was observed in the gastric surface epithelium, and this staining was focused in the nucleus; C, D: Strongly apoptotic cells were observed in the nucleus of the gastric surface epithelium. Similar to the cell proliferation, the cell apoptosis was significantly different between the RP and XP groups at 3 and 6 h ( $P < 0.05$ ). Original magnification  $\times 400$ . <sup>a</sup> $P < 0.05$  vs the model group (MP group); <sup>c</sup> $P < 0.05$  vs the control group (CP group).

inhibiting NF- $\kappa$ B-mediated nitric oxide and pro-inflammatory cytokine production<sup>[60]</sup>. Except these effects, the XTTF granule has also been shown to play a role in the manipulation of HSP70 expression, and although the data are still limited, the previous study did indicate that some herbal medicine constituents, such as *Glycyrrhizae Radix Et Rhizoma* (an herbal component in the Xiao-cheng-qi decoction<sup>[19]</sup>), could promote HSP expression<sup>[61]</sup>. Interestingly, *Glycyrrhizae Radix Et Rhizoma* was also demonstrated to be effective in protecting gastric mucosa *via* gastric mucin<sup>[62]</sup>. Finally, the XTTF granule also inhibited cell apoptosis and promoted cell proliferation, which is related to the mucosal protection of some components such as *Magnoliae Officinalis Cortex*<sup>[62]</sup> and *Aurantii Fructus Immaturus*<sup>[63]</sup>. We speculate that all of these actions may contribute to tissue regeneration and reconstruction in the stomach<sup>[64]</sup>.

It should be noted that the parameters manipulated by the XTTF granule in SU might not work individually, and these parameters could be connected in a complex relationship. For example, previous studies have shown that the aforementioned MIF inhibition effect of the

XTTF granule could result in the elevation of nitric oxide levels<sup>[57]</sup>, which are involved in HSP70 expression<sup>[65]</sup> and cell proliferation<sup>[66]</sup> during ulcer healing in the stomach. HSP70 could also exert its cytoprotective effect by interfering with the stress-induced apoptotic pathway<sup>[67,68]</sup>. Except that studies have indicated that ranitidine can inhibit gastric acid secretion<sup>[12]</sup>, accelerate GER<sup>[33,42]</sup>, and reduce apoptosis levels<sup>[69]</sup>, our study showed that the effect of ranitidine on parameters such as promoting cell proliferation may also be attributed to the comprehensive network of SU. The XTTF granule was shown to prevent ulcers and promote healing by attenuating aggressive factors and enhancing defensive factors. Future studies, such as randomized controlled trials, are necessary to further confirm its efficacy.

This study has several limitations. First, although we demonstrated that the XTTF granule exerts measurable preventative effects on SU, whether the XTTF granule acts in a dose-dependent manner remains unknown. Second, pretreatment with the XTTF granule in rats scheduled to undergo stress may not correspond to clinical practice because the majority of patients are administered

pharmacological agents for SUP after stress. Additional studies are necessary to measure the efficacy of the XTTF granule in this scenario.

## COMMENTS

### Background

Stress ulcer prophylaxis plays a pivotal role in the care of critically ill patients. Recent studies indicated that traditional Chinese medicine (TCM) could exert measurable therapeutic effects on gastric ulcers. The Xiaotan Tongfu (XTTF) granule has been used for a long time to treat gastrointestinal disorders in critically ill patients. However, whether it could be applied to stress ulcers remained unknown.

### Research frontiers

Emerging evidence suggests that TCM was effective in the management of stress-related gastrointestinal disorders, such as irritable bowel syndrome. In addition, a number of studies have also indicated that TCM could exert measurable therapeutic effects on gastric ulcers in rats. Stress ulceration was an inevitable complication of the gastrointestinal tract in animals experiencing abnormally high physiological stress. In this study, the authors demonstrated that a traditional Chinese herbal decoction could play an important role in the prevention of stress ulcers.

### Innovations and breakthroughs

Although the underlying mechanism of stress ulcers was commonly believed to depend on the balance between known aggressive factors and mucosal defense mechanisms, most of the clinical strategies are still aimed at inhibiting gastric acid. This study focused on demonstrating the efficacy of a traditional Chinese herbal medicine used to treat stress ulcers. The study indicated that traditional Chinese herbal medicine was effective in preventing stress ulcers and that inhibiting gastric acid would not be the only strategy.

### Applications

By confirming its efficacy and potential mechanisms in an animal study, the findings suggest that the XTTF granule could be regarded as a potential option for stress ulcer prophylaxis in the future.

### Terminology

Stress ulceration refers to an inevitable complication of the gastrointestinal tract in people experiencing abnormally high physiological stress, which usually leads to gastrointestinal bleeding. The underlying mechanism of stress ulcers was commonly believed to depend on the balance between known aggressive factors and mucosal defense mechanisms. Therefore, any agents that can interfere with the above factors, either individually or collectively, could be used as a stress ulcer prophylaxis.

### Peer review

The authors examined the efficacy and potential mechanisms of the XTTF granule in stress ulcers. This study revealed that the XTTF granule was similar to ranitidine treatment with regard to reducing gastric lesions in a cold-restraint stress model, and the underlying mechanisms may include acceleration of the gastric emptying rate, inhibition of local inflammation, promotion of cell proliferation and suppression of apoptosis. The results are interesting and may represent a potential option in the management of stress ulcers in the future.

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## Propofol induces apoptosis and increases gemcitabine sensitivity in pancreatic cancer cells *in vitro* by inhibition of nuclear factor- $\kappa$ B activity

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### Abstract

**AIM:** To investigate the effect of propofol on human pancreatic cells and the molecular mechanism of propofol action.

**METHODS:** We used the human pancreatic cancer cell line MIAPaCa-2 for *in vitro* studies measuring growth inhibition and degree of apoptotic cell death induced by propofol alone, gemcitabine alone, or propofol followed by gemcitabine. All experiments were conducted in triplicate and carried out on three or more separate occasions. Data were means of the three or more independent experiments  $\pm$  SE. Statistically significant differences were determined by two-tailed unpaired Student's *t* test and defined as  $P < 0.05$ .

**RESULTS:** Pretreatment of cells with propofol for 24 h followed by gemcitabine resulted in 24%-75% growth inhibition compared with 6%-18% when gemcitabine was used alone. Overall growth inhibition was directly correlated with apoptotic cell death. We also showed that propofol potentiated gemcitabine-induced killing by downregulation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). In con-

trast, NF- $\kappa$ B was upregulated when pancreatic cancer cells were exposed to gemcitabine alone, suggesting a potential mechanism of acquired chemoresistance.

**CONCLUSION:** Inactivation of the NF- $\kappa$ B signaling pathway by propofol might abrogate gemcitabine-induced activation of NF- $\kappa$ B, resulting in chemosensitization of pancreatic tumors to gemcitabine.

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**Key words:** Pancreatic cancer; Propofol; Gemcitabine; Nuclear factor- $\kappa$ B; Apoptosis

**Core tip:** Pretreatment of cells with propofol for 24 h followed by gemcitabine resulted in significant growth inhibition compared with gemcitabine alone. Overall growth inhibition correlated directly with apoptotic cell death. Propofol potentiated gemcitabine-induced killing by downregulation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). In contrast, NF- $\kappa$ B was upregulated when pancreatic cancer cells were exposed to gemcitabine alone. These results suggested that inactivation of the NF- $\kappa$ B signaling pathway by propofol abrogated gemcitabine-induced activation of NF- $\kappa$ B resulting in the chemosensitization of pancreatic tumors to gemcitabine.

Du QH, Xu YB, Zhang MY, Yun P, He CY. Propofol induces apoptosis and increases gemcitabine sensitivity in pancreatic cancer cells *in vitro* by inhibition of nuclear factor- $\kappa$ B activity. *World J Gastroenterol* 2013; 19(33): 5485-5492 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5485.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5485>

### INTRODUCTION

Pancreatic cancer has the poorest prognosis of all major cancers, with an overall 5-year survival rate of around 5%<sup>[1]</sup>. The current clinical standard of care for advanced pancre-

atic cancer is gemcitabine, a cytotoxic nucleoside analog. Treatment with gemcitabine results in a tumor response rate of 12% and a median survival time of 5 mo<sup>[2]</sup>.

Drug resistance (both intrinsic and acquired) is thought to be a major reason for the limited benefit of most pancreatic cancer therapies<sup>[3]</sup>. Recent studies have indicated that targeted therapies in combination with gemcitabine can have statistically significant benefits<sup>[4]</sup>. However, the results to date remain insufficient, and new approaches to improving the effectiveness of gemcitabine are needed. One of the targets considered for combination therapy that has received wide attention is the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B)<sup>[5]</sup>. Pan *et al*<sup>[6]</sup> and Kong *et al*<sup>[7]</sup> reported that inhibition of NF- $\kappa$ B might be useful for pancreatic cancer therapy, as it increases gemcitabine sensitivity in pancreatic cancer cells. Recent studies also indicate that combination therapy with a targeted medicine that inhibits NF- $\kappa$ B activity potentiated the anti-tumor effects of gemcitabine in pancreatic cancer cells<sup>[8-10]</sup>.

Propofol is an intravenous anesthetic that is used to induce and maintain anesthesia, and to sedate and calm patients in intensive care. Increasing evidence suggests that propofol might be neuroprotective against ischemic neuronal injury in animal models of cerebral ischemia<sup>[11-13]</sup>. Xi *et al*<sup>[14]</sup> and Li *et al*<sup>[15]</sup> found that the neuroprotective effects of propofol against neuronal apoptosis might be a consequence of regulation of Bcl-2, caspase-3 and Bax. Propofol has protective effects against digestive injury. It inhibits HMGB1 expression and TLR4/MyD88/NF- $\kappa$ B-mediated inflammatory responses, and hampers apoptosis, which might contribute to its protective action against ethanol-induced gastric mucosal injury<sup>[16]</sup>. Propofol also has anticancer properties. Siddiqui *et al*<sup>[17]</sup> found that combinations of propofol and docosahexaenoate or propofol and eicosapentaenoate significantly induced apoptosis and inhibited cell adhesion and migration in breast cancer cells. Propofol inhibits MMP-2 and -9 expression, suppressing lung cancer cell invasion and migration<sup>[18]</sup>. Propofol induces proliferation and promotes invasion of gallbladder cancer cells through activation of Nrf2<sup>[19]</sup>. Li *et al*<sup>[20]</sup> showed that propofol reduced the level of MMP in breast cancer cells by inhibition of NF- $\kappa$ B pathways, significantly restraining migration and invasion of breast cancer cells. Propofol extensively counteracts the oxidative/nitrative and multiple apoptotic effects of doxorubicin in rat hearts<sup>[21]</sup>.

Most human pancreatic tumors show high levels of activated NF- $\kappa$ B, which mediates survival signaling and confers resistance to conventional therapeutics. Therefore, targeting NF- $\kappa$ B could be an effective therapeutic approach. The mechanism by which NF- $\kappa$ B stimulates cell survival is not fully understood; however, recent studies showed that activation of NF- $\kappa$ B leads to the activation of a series of survival factors, including bcl-2. This allows cancer cells to resist induction of apoptosis<sup>[7]</sup>. Many conventional cancer chemotherapeutic agents such as vinblastine, vincristine, daunomycin, doxorubicin, camptothecin, cisplatin, and etoposide activate NF- $\kappa$ B. This activation results in resistance to apoptosis, which results in poor clinical outcomes for pancreatic cancer patients.

Inactivating NF- $\kappa$ B activity induces apoptosis and abrogates *de novo* or acquired chemoresistance<sup>[22]</sup>. Based on these results, we hypothesized that propofol might block multiple intracellular signaling pathways that are known to confer a high degree of chemoresistance by pancreatic cancer cells, abrogating either *de novo* or acquired chemoresistance. Although the development of alternative gemcitabine schedules and chemotherapy combinations continues, we report our observations in support of our hypothesis that better pancreatic cancer cell killing is feasible by using propofol with gemcitabine. Our results are primarily due to inactivation of NF- $\kappa$ B signaling *in vitro*.

## MATERIALS AND METHODS

### Cell culture

The MIA-PaCa-2 human pancreatic cancer cell line was obtained from the American Type Culture Collection and cultured in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, L-glutamine, penicillin/streptomycin antibiotics, and vitamins. Cells were maintained in a humidified incubator containing 10% carbon dioxide at 37 °C. Cells underwent serum starvation for 24 h before treatment with propofol or/and gemcitabine.

### Drug treatment

For single-agent treatment, MIA-PaCa-2 cells were treated with 10-100  $\mu$ mol/L propofol or 0.5 mmol/L Na<sub>2</sub>CO<sub>3</sub> (vehicle control) for 72 h; 100  $\mu$ mol/L per milliliter propofol for 24, 48, 72 h; or 10, 25, 50, 100  $\mu$ mol/L gemcitabine for 72 h. For combined treatment, MIA-PaCa-2 cells were treated with 50 or 100  $\mu$ mol/L per milliliter propofol for 24 h, then exposed to 10-100  $\mu$ mol/L gemcitabine for an additional 72 h.

### Growth inhibition by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

MIA-PaCa-2 cells were seeded at  $3 \times 10^3$  cells per well in 96-well microtiter culture plates. After overnight incubation, medium was replaced with fresh medium containing propofol 0-100  $\mu$ mol/L diluted from a 10 mmol/L stock. After 24-72 h incubation, 20  $\mu$ L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added to each well and incubated for 2 h. Supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100  $\mu$ L isopropanol. Plates were mixed for 30 min on a shaker and absorbance was measured at 595 nm using a plate reader (TECAN, Durham, NC, United States). MIA-PaCa-2 cells were also treated with 25  $\mu$ mol/L propofol for 24 h and exposed to 0-100  $\mu$ mol/L gemcitabine for an additional 72 h before MTT assay.

### DNA ladder analysis for apoptosis

Cytoplasmic DNA was extracted from MIA-PaCa-2 cells treated with 100  $\mu$ mol/L propofol or 0.5 mmol/L Na<sub>2</sub>CO<sub>3</sub> (vehicle control) for 24-72 h; or 10-100  $\mu$ mol/L propo-

fol for 72 h using 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, and 0.2% Triton X-100. MIA-PaCa-2 cells were also treated with 25  $\mu\text{mol/L}$  propofol for 24 h and exposed to 0-100  $\mu\text{mol/L}$  of gemcitabine for an additional 72 h before cytoplasmic DNA extraction. Lysate was centrifuged for 15 min at 13000 *g* to separate fragmented DNA (soluble) from intact chromatin (nuclear pellet). Supernatant from lysates was treated with RNase followed by SDS-Proteinase K digestion, phenol chloroform extraction, and isopropanol precipitation. DNA was separated by 1.5% agarose gels stained with ethidium bromide for DNA visualization by UV light.

#### **Terminal transferase dUTP nick-end labeling assay for apoptosis**

Apoptosis was evaluated by terminal transferase dUTP nick-end labeling (TUNEL) assay according to the manufacturer's instructions for MIA-PaCa-2 cells treated with 100  $\mu\text{mol/L}$  propofol or 0.5 mmol/L  $\text{Na}_2\text{CO}_3$  (vehicle control) for 24-72 h; or 10-100  $\mu\text{mol/L}$  propofol for 72 h; or 25  $\mu\text{mol/L}$  propofol for 24 h followed by 0-100  $\mu\text{mol/L}$  of gemcitabine for 72 h. TUNEL-positive cells were colored using diaminobenzidine as chromogen and counterstained with hematoxylin. The percentage of TUNEL-positive cells was assessed in five randomly selected fields per section. All assays were performed in quadruplicate.

#### **Quantification of apoptosis by enzyme-linked immunosorbent assay**

The Cell Apoptosis enzyme-linked immunosorbent assay (ELISA) Detection Kit (Chemicon International, Temecula, CA, United States) was used to detect apoptosis in MIA-PaCa-2 cells according to the manufacturer's protocol. MIA-PaCa-2 cells were treated with 10-100  $\mu\text{mol/L}$  propofol for 72 h or with 50  $\mu\text{mol/L}$  propofol for 24-72 h; or 50  $\mu\text{mol/L}$  propofol for 24 h followed by 0-100  $\mu\text{mol/L}$  gemcitabine for 72 h. After treatment, cytoplasmic histone DNA fragments from MIA-PaCa-2 cells were extracted and bound to immobilized anti-histone. Peroxidase-conjugated anti-DNA was used to detect immobilized histone DNA fragments. After addition of peroxidase substrate, spectrophotometric absorbance of samples was determined using an ULTRA Multifunctional Microplate Reader (TECAN) at 405 nm.

#### **Electrophoretic mobility shift assay**

Cell extracts were prepared using a commercially available nuclear extraction kit according to the manufacturer's protocol (Pierce, Rockford, IL, United States). Electrophoretic mobility shift assay (EMSA) was performed according to the provided protocol (Promega). Briefly, cells were washed with cold PBS and suspended in 0.15 mL lysis buffer (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 2  $\mu\text{g/mL}$  leupeptin, 2  $\mu\text{g/mL}$  aprotinin, and 0.5 mg/mL benzamidine). Cells were swelled on ice for 20 min and 4.8  $\mu\text{L}$  10% NP40 was added. Tubes

were vigorously mixed for a few seconds and microcentrifuged. The nuclear pellet was resuspended in 30  $\mu\text{L}$  ice-cold nuclear extraction buffer (20 mmol/L HEPES pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 2  $\mu\text{g/mL}$  leupeptin, 2  $\mu\text{g/mL}$  aprotinin, and 0.5 mg/mL benzamidine) and incubated on ice with intermittent mixing. Tubes were microcentrifuged for 5 min at 4  $^{\circ}\text{C}$ , and supernatant (nuclear extract) was collected in cold Eppendorf tubes and stored at -70  $^{\circ}\text{C}$ . Protein content was measured by bicinchoninic acid method. EMSA used 5  $\mu\text{g}$  of nuclear proteins incubated with IRDye-700-labeled NF- $\kappa\text{B}$  oligonucleotide. Incubation mixture was 2  $\mu\text{g}$  of poly (deoxyinosinic - deoxycytidylic acid) in binding buffer. DNA-protein complexes were separated from free oligonucleotides on 8.0% native polyacrylamide gels using buffer containing 50 mmol/L Tris, 200 mmol/L glycine (pH 8.5), and 1 mmol/L EDTA and visualized by an Odyssey Infrared Imaging System using Odyssey Software Release 1.1. Equal protein loading was ensured by immunoblotting 10  $\mu\text{g}$  of nuclear protein with anti-retinoblastoma.

#### **Statistical analysis**

All experiments were conducted in triplicate and carried out on three or more separate occasions. Data were means of the three or more independent experiments  $\pm$  SE. Statistically significant differences were determined by two-tailed unpaired Student's *t* test and defined as  $P < 0.05$ .

## **RESULTS**

#### **Effect of propofol on cell proliferation**

MIA-PaCa-2 cells were treated with 0-100  $\mu\text{mol/L}$  propofol over 72 h, and cell viability was determined by MTT assay. Treatment with 10, 25, 50, or 100  $\mu\text{mol/mL}$  of propofol for 72 h resulted in 95%, 87%, 64%, and 51% of cell growth relative to control, respectively (Figure 1A). Similar results were found with exposure to 100  $\mu\text{mol/mL}$  propofol for 24, 48, and 72 h (Figure 1B). Treatment of MIA-PaCa-2 cells with propofol resulted in dose- and time-dependent inhibition of cell proliferation, demonstrating that propofol applied as a single agent was an effective inhibitor of pancreatic cancer cell growth.

#### **Effect of propofol on apoptosis**

MIA-PaCa-2 cells were treated with 10-100  $\mu\text{mol/L}$  propofol for 72 h, or 100  $\mu\text{mol/L}$  propofol for 24-72 h. Apoptosis was determined by TUNEL, DNA ladder and ELISA assays. Treatment with 10, 25, 50, or 100  $\mu\text{mol/mL}$  propofol for 72 h resulted in 1.6%, 4.1%, 9.7%, or 13.8% apoptosis relative to controls (Figure 2A). Similar results were found with 100  $\mu\text{mol/mL}$  propofol for 24, 48, or 72 h (data not shown). Treatment of MIA-PaCa-2 cells with propofol resulted in dose- and time-dependent promotion of apoptosis, demonstrating that propofol used as a single agent was an effective promoter of pancreatic cancer cell death. DNA ladder (Figure 2B) and ELISA (Figure 2C)

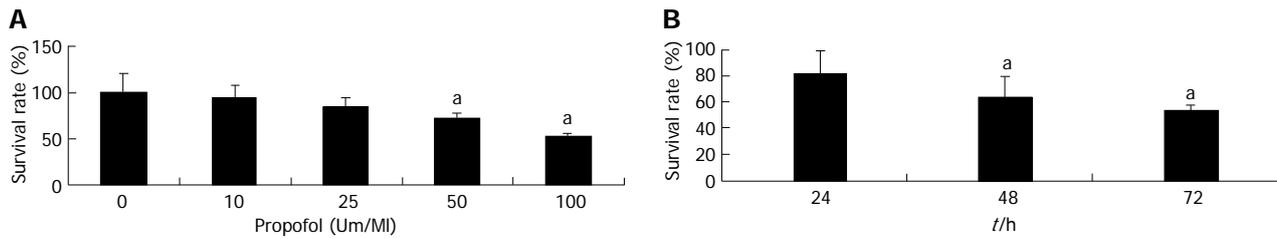


Figure 1 Evaluation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of pancreatic cancer MIA-PaCa-2 cell viability after propofol pretreatment. A: Cells were either untreated or treated with 10-100  $\mu\text{mol/mL}$  propofol for 72 h; B: Cells treated with 100  $\mu\text{mol/mL}$  propofol for 24, 48, or 72 h. <sup>a</sup> $P < 0.05$  vs control group.

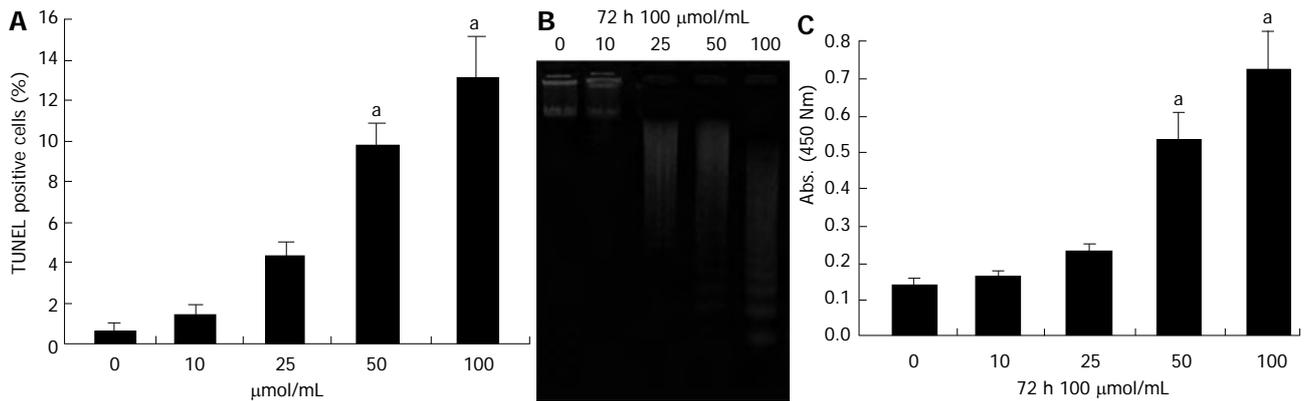


Figure 2 Evaluation of apoptosis of pancreatic cancer MIA-PaCa-2 cells after propofol treatment using terminal transferase dUTP nick-end labeling, DNA ladder and enzyme-linked immunosorbent assays. A: Propofol-induced apoptotic cell death by terminal transferase dUTP nick-end labeling (TUNEL) after 72 h of 10-100  $\mu\text{mol/L}$  propofol; B: DNA ladder indicative of apoptosis in pancreatic cancer cells treated with 10-100  $\mu\text{mol/mL}$  propofol for 72 h. C: Propofol-induced apoptosis measured by enzyme-linked immunosorbent assay after 72 h of 10-100  $\mu\text{mol/L}$  propofol. <sup>a</sup> $P < 0.05$  vs control group.

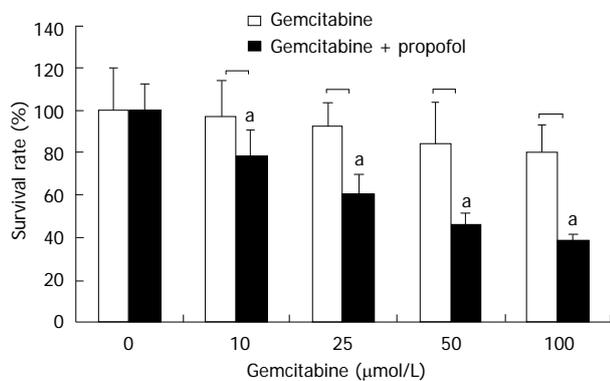


Figure 3 Cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of pancreatic cancer MIA-PaCa-2 cells after propofol pretreatment. MIA-PaCa-2 cells pretreated with propofol (50  $\mu\text{mol/mL}$ ) for 24 h followed by cocubation with gemcitabine (10, 25, 50 and 100  $\mu\text{mol/L}$ ) for 72 h were analyzed for viable cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Viable cells were evaluated relative to gemcitabine-treated controls and interpreted as % viable cells. Data are averages of four to five independent experiments. <sup>a</sup> $P < 0.05$  vs control group.

assays gave the same results as TUNEL assays.

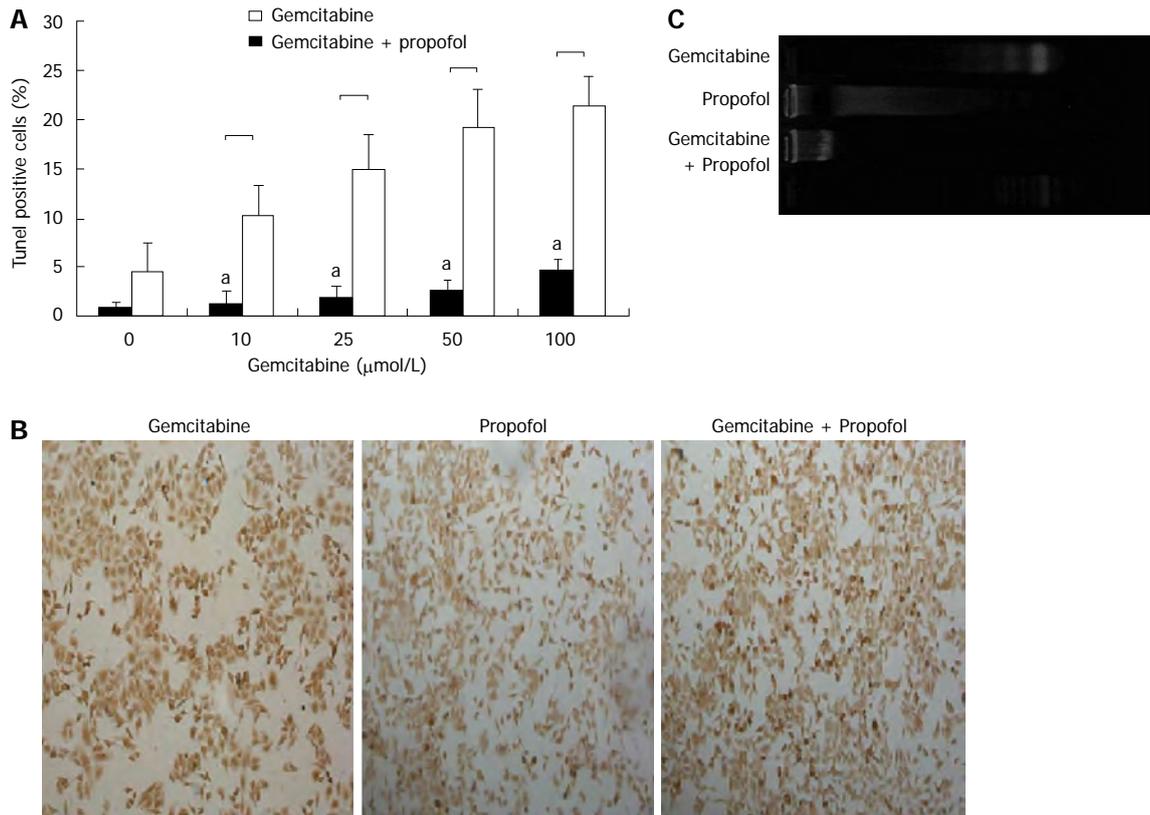
### Propofol potentiates growth inhibition by gemcitabine in MIA-PaCa-2 cells

MIA-PaCa-2 cells are resistant to gemcitabine treatment. We found that gemcitabine treatment did not result in

obvious MIA-PaCa-2 growth inhibition (Figure 3). We assessed the effect on cell viability of pretreatment and cotreatment of propofol and gemcitabine by MTT assay. Cells were pretreated with propofol (50  $\mu\text{mol/mL}$ ) alone or in combination with a single dose of gemcitabine (10, 25, 50 and 100  $\mu\text{mol/L}$ ), and viable cells were evaluated by MTT assay 72 h after treatment. Doses were chosen based upon a preliminary dose escalation study (data not shown). Treatment of MIA-PaCa-2 cells with a single dose of gemcitabine (10, 25, 50 and 100  $\mu\text{mol/L}$ ) for 72 h resulted in only 6% to 18% loss of viability. However, pretreatment with propofol for 24 h followed by treatment with gemcitabine resulted in 24% to 75% loss of viable MIA-PaCa-2 cells (Figure 3). These results suggested that the combination of propofol with low therapeutic doses of gemcitabine elicited significantly greater inhibition of cancer cell growth compared with either agent alone. This suggested that lower toxic side effects are likely to occur in normal cells. Inhibition of cell growth and viability as assessed by MTT assay might be due to the induction of apoptosis by propofol and gemcitabine. We therefore investigated whether gemcitabine in combination with propofol induced more apoptosis than either agent alone.

### Propofol sensitizes MIA-PaCa-2 cells to apoptosis induced by gemcitabine

By TUNEL analysis, treatment of MIA-PaCa-2 cells with



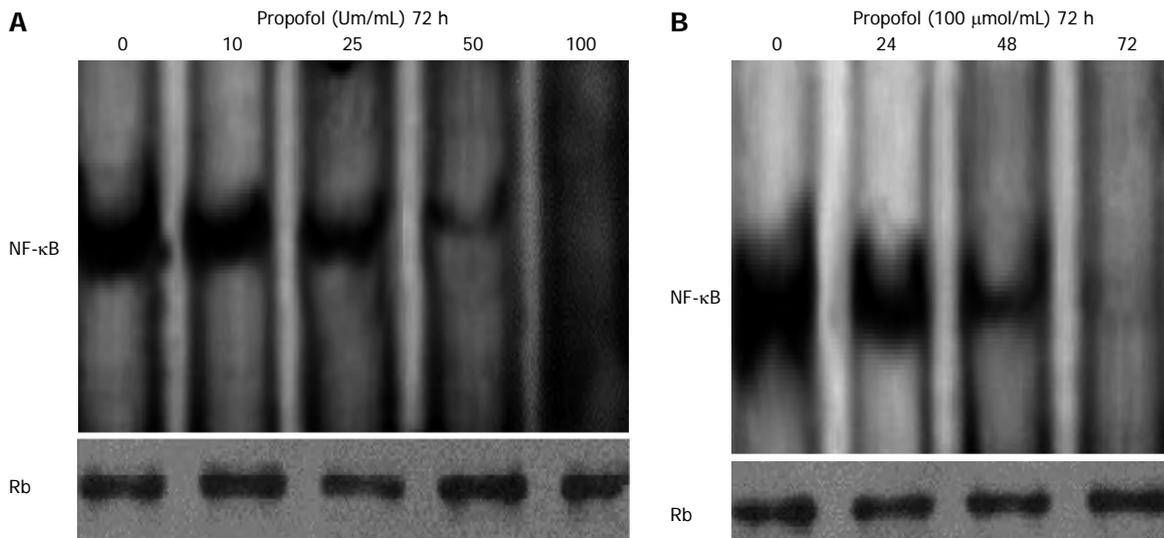
**Figure 4** Evaluation of apoptosis by terminal transferase dUTP nick-end labeling and DNA ladder assays in pancreatic cancer MIA-PaCa-2 cells after propofol pretreatment. **A:** Sensitization of pancreatic tumor MIA-PaCa-2 cells to propofol- and/or gemcitabine-induced apoptosis measured by terminal transferase dUTP nick-end labeling (TUNEL) assay after 24 h of pretreatment with propofol (50 μmol/mL), gemcitabine (0-100 μmol/L), or propofol and gemcitabine combined for 72 h. Increased apoptosis was evident in the combination treatment group relative to individual treatment groups. <sup>a</sup>*P* < 0.05 vs propofol and gemcitabine combined group; **B:** Representative TUNEL image of MIA-PaCa-2 cells pretreated with propofol (50 μmol/mL) for 24 h followed by coincubation with gemcitabine (50 μmol/L) for 72 h; **C:** Representative DNA ladder image of MIA-PaCa-2 cells pretreated with propofol (50 μmol/mL) for 24 h followed by coincubation with gemcitabine (50 μmol/L) for 72 h.

propofol (25 μmol/mL) for 72 h resulted in only 4.6% apoptosis (Figure 2 A), and treatment with 10, 25, 50 and 100 μmol/L gemcitabine) for 72 h resulted in 1.2% to 4.6% apoptosis (Figure 4A). Only low levels of apoptosis were detected with single-agent treatment. Figure 4B has representative TUNEL data of MIA-PaCa-2 cells pretreated with propofol (50 μmol/mL) for 24 h followed by coincubation with gemcitabine (25 μmol/L) for 72 h. Apoptosis measured by ELISA and DNA ladder assays gave the same results (data not shown). Figure 4C has Representative DNA ladder assay data of MIA-PaCa-2 cells pretreated with propofol (50 μmol/mL) for 24 h followed by coincubation with gemcitabine (25 μmol/L) for 72 h.

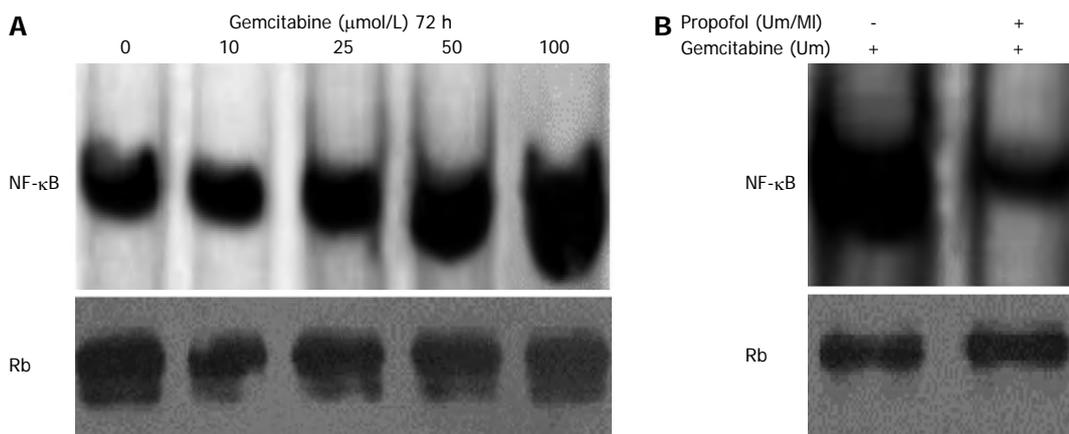
MIA-PaCa-2 cells pretreated with propofol (25 μmol/mL) for 24 h, followed by coincubation with gemcitabine (10, 25, 50 and 100 μmol/L) for 72 h, showed significant cell apoptosis by DNA ladder, TUNEL assay and ELISA assay. These results were consistent with the growth inhibition MTT assays, and suggested that loss of viable cells by propofol and gemcitabine was partly due to the induction of apoptosis.

#### Propofol inhibits NF-κB DNA-binding activity

Consistent with earlier reports, constitutively active NF-κB DNA-binding activity was found in nuclear extracts from MIA-PaCa-2 cells. Band specificity was confirmed by supershift. Based on reports indicating the potential of propofol to abrogate constitutive and inducible NF-κB in halothane-induced rat liver<sup>[23]</sup>, we analyzed whether propofol abrogated basal constitutive activation of NF-κB in MIA-PaCa-2 cells. To evaluate the effect of propofol in MIA-PaCa-2 cells, semiconfluent cells were treated with 0, 10, 25, 50 or 100 μmol/mL propofol for 72 h. As shown in Figure 5A, incubation with 50 μmol/mL propofol for 72 h resulted in a significant decrease in NF-κB DNA-binding activity in the MIA-PaCa-2 cells, and incubation with 100 μmol/mL propofol for 72 h resulted in complete disappearance of the activity. MIA-PaCa-2 cells treated with 100 μmol/L propofol for 24-72 h resulted in gradually reduced NF-κB DNA-binding activity (Figure 5B). These results clearly suggested that propofol was effective at downregulating NF-κB DNA-binding activity. We found no alterations in the nuclear protein content of retinoblastoma, which was used as protein loading control.



**Figure 5** Propofol inhibits constitutively active nuclear factor-κB in MIA-PaCa-2 cells. A: MIA-PaCa-2 cells were treated with 0, 10, 25, 50, or 100 μmol/mL propofol for 72 h and nuclear extracts were probed for nuclear factor-κB (NF-κB) binding to a DNA consensus sequence. Results of NF-κB DNA-binding activity by electrophoretic mobility shift assay (EMSA); B: MIA-PaCa-2 cells were treated with 100 μmol/mL propofol for 24, 48, or 72 h and nuclear extracts were probed for NF-κB binding to a DNA consensus sequence. Results of NF-κB DNA-binding activity by EMSA. Retinoblastoma protein in the nuclear extract was used as a loading control.



**Figure 6** Propofol abrogates gemcitabine-induced nuclear factor-κB in MIA-PaCa-2 cells. A: MIA-PaCa-2 cells were exposed to 10-100 μmol/L gemcitabine for 72 h. Nuclear extracts were analyzed by electrophoretic mobility shift assay (EMSA); B: Propofol abrogated gemcitabine induced nuclear factor-κB (NF-κB) in MIA-PaCa-2 cells exposed to 100 μmol/mL propofol for 24 h followed by incubation with 100 μmol/L gemcitabine. Nuclear extracts were harvested at 72 h and analyzed by EMSA. Propofol pretreatment downregulated gemcitabine-induced NF-κB with 100 μmol/L gemcitabine.

**Propofol abrogates NF-κB activation induced by gemcitabine**

Next, we analyzed whether gemcitabine induced NF-κB DNA-binding activity and whether inactivation of NF-κB by propofol abrogated the chemoresistant phenotype of MIA-PaCa-2 cells, resulting in more pronounced gemcitabine-induced apoptosis. First, we analyzed dose and time responses to gemcitabine by induction of NF-κB in MIA-PaCa-2 cells. Nuclear extracts were prepared from MIA-PaCa-2 cells treated with 10, 25, 50 or 100 μmol/L gemcitabine for up to 72 h and analyzed for NF-κB DNA-binding activity by EMSA. Our results showed dose escalation of gemcitabine, with significant upregulation of constitutive NF-κB DNA-binding activity after gemcitabine treatment (Figure 6A). Pretreatment of cells with 100 μmol/mL propofol for 24 h abrogated

gemcitabine-induced activation of NF-κB DNA-binding activity (Figure 6B). These results showed that propofol downregulates NF-κB DNA-binding activity in unstimulated conditions and inhibits gemcitabine-induced NF-κB activity. This might be the molecular mechanism of gemcitabine-induced cell death in propofol-pretreated cells.

**DISCUSSION**

Despite rapid advances in diagnostic and operative techniques, pancreatic cancer remains one of the most difficult human malignancies to treat. This is partly due to the advanced stage of the disease and *de novo* chemoresistant behavior towards cytotoxic chemotherapeutic agents and/or radiotherapy. In recent years, this problem has been addressed by a combinatorial approach. Several

randomized studies have shown a significant increase in patient response rates when different classes of chemotherapeutic agents are used in combination. However, a major problem is high treatment-associated toxicity with no added benefit in significant overall survival<sup>[24-26]</sup>. The FOLFIRINOX regimen (bolus and infusional 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin) is a new option for patients with metastatic pancreatic cancer and has good performance. Compared with gemcitabine, FOLFIRINOX was associated with a survival advantage, but had increased toxicity<sup>[27]</sup>. These limitations could be overcome by rational chemotherapeutic combinations in which toxic agents are used in lower doses, and treatment efficacy is increased by use of a nontoxic agent with a different mechanism of action.

Propofol is a fairly new induction agent introduced in the 1980s. Animal studies have demonstrated the neuroprotective ability of propofol. Recent studies of propofol effects during global cerebral ischemia-reperfusion injury in rats (4-vessel occlusion) concluded that propofol inhibited neuronal death induced by brain ischemia<sup>[10,14,28]</sup>. Propofol is also a glutamate antagonist at the NMDA-receptor level and a calcium-channel antagonist<sup>[29]</sup>, has GABAergic activity and antioxidant properties<sup>[29]</sup>, and reduces excitotoxicity<sup>[30]</sup>. Increasing evidence suggests that propofol has anticancer properties<sup>[17-20]</sup>.

In this study, we used propofol in combination with a commonly used chemotherapeutic agent, gemcitabine, and tested its efficacy against the pancreatic cancer cell line MIA-PaCa-2. This preclinical study documented that sensitization of cancer cells was achieved by propofol during gemcitabine-induced killing, as shown by more pronounced cell death compared with single-agent treatment. We found that propofol pretreatment significantly enhanced tumor cell killing compared with either agent alone. This observation is important because 75% growth inhibition could be achieved using the same doses of gemcitabine that produced only 18% growth inhibition when gemcitabine was used alone. Consistent with the previously observed apoptotic effect of propofol, we showed that propofol alone not only significantly promoted pancreatic cancer MIA-PaCa-2 cell death and apoptosis, but also promoted gemcitabine-induced apoptosis as determined by MTT, ELISA, TUNEL staining and DNA ladder assays. In addition, we found for the first time that propofol inhibited the NF- $\kappa$ B activity in the MIA-PaCa-2 cells as demonstrated by EMSA assay. Together, these observations suggested that propofol strongly sensitized pancreatic cancer cells to gemcitabine-induced apoptosis.

Our results also showed that gemcitabine alone activated NF- $\kappa$ B, resulting in reduced apoptosis. This supported the model that NF- $\kappa$ B activation inhibits apoptosis. In addition, our *in vitro* results showed that propofol alone or propofol pretreatment followed by gemcitabine treatment abrogated NF- $\kappa$ B activation and increased the apoptotic index, suggesting that inhibition of NF- $\kappa$ B is mechanistically associated with sensitization of pancreatic cancer cells to apoptosis.

In conclusion, our findings are consistent with the hypothesis that chemosensitivity of pancreatic cancer cells is enhanced by pretreatment with propofol and that this effect is mediated by inactivation of NF- $\kappa$ B DNA-binding activity leading to apoptosis. Although in clinical practice the use of propofol in combination with gemcitabine might not result in this enhancement, our findings provide a new insight into propofol in cancer treatment. Our results suggest that propofol can be an anesthetic agent that reduces pain and might also be important in inhibiting the growth of pancreatic cancer cells in pancreatic cancer therapy.

## COMMENTS

### Background

Propofol is a popular agent for anesthesia and long-term sedation. Recently, propofol was found to be effective at inducing apoptosis and possibly contributing to anti-tumor activity.

### Research frontiers

Propofol was found to effectively induce apoptosis and possibly contribute to anti-tumor activity. Research is needed on whether propofol might inhibit growth, promote apoptosis, and increase gemcitabine sensitivity in pancreatic cancer cells.

### Innovations and breakthroughs

Propofol has anticancer properties. The combination of propofol and docosahexaenoate or propofol and eicosapentaenoate might significantly induce apoptosis and inhibit cell adhesion and migration in breast cancer cells. The results of this study show that pretreatment of cells with propofol for 24 h followed by gemcitabine resulted in 24%-75% growth inhibition compared with 6%-18% for gemcitabine used alone. Overall growth inhibition was directly correlated with apoptosis. The authors also showed that propofol potentiated gemcitabine-induced killing by downregulation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). In contrast, NF- $\kappa$ B was upregulated when pancreatic cancer cells were exposed to gemcitabine alone, suggesting a potential mechanism of acquired chemoresistance.

### Applications

This study suggests that inactivation of the NF- $\kappa$ B signaling pathway by propofol could abrogate gemcitabine-induced activation of NF- $\kappa$ B, resulting in the chemosensitization of pancreatic tumors to gemcitabine.

### Terminology

Propofol is an intravenous anesthetic used to induce and maintain anesthesia, and to sedate and calm patients in intensive care units.

### Peer review

In this experimental study, the authors show that propofol induces apoptosis and increases *in vitro* gemcitabine sensitivity in pancreatic cancer cells by inhibition of NF- $\kappa$ B activity. The manuscript is interesting.

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## Bisacodyl plus split 2-L polyethylene glycol-citrate-simethicone improves quality of bowel preparation before screening colonoscopy

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### Abstract

**AIM:** To compare the bowel cleansing efficacy, tolerability and acceptability of split 2-L polyethylene glycol (PEG)-citrate-simethicone (PEG-CS) plus bisacodyl (BIS) vs 4-L PEG for fecal occult blood test-positive screening colonoscopy.

**METHODS:** This was a randomised, observer-blind comparative study. Two hundred and sixty-four subjects underwent screening colonoscopy (mean age  $62.5 \pm 7.4$  years, male 61.7%). The primary objective of the study was to compare the bowel cleansing efficacy of the two preparations. Interventions: BIS plus PEG-CS: 3 tablets of 5-mg BIS at 16:00, PEG-CS 1-L at 19:00 and 1-L at 7:00, 4-L PEG: 3-L at 17:00, and 1-L at 7:00. Colonoscopy was carried out after 11:00, at least 3 h after the completion of bowel preparation. Bowel cleansing was evaluated using the Harefield Cleansing Scale.

**RESULTS:** Bowel preparation was successful for 92.8% of subjects in the PEG-CS group and for 92.1% of subjects in the 4-L PEG (RR = 1.01; 95%CI: 0.94-1.08). BIS + PEG-CS was better tolerated than 4-L PEG. A

greater rate of patients in the BIS + PEG-CS group had no difficulty and/or were willing to repeat the same preparation compared to split-dose 4-L PEG group. Subjects in the BIS + PEG-CS group rated the prep as good or satisfactory in 90.6% as compared to 77% in the 4-L PEG ( $P = 0.003$ ). Subjects receiving BIS + PEG-CS stated they fully adhered to instructions drinking all the 2-L solution in 97.1% compared with 87.3% in the 4-L PEG ( $P = 0.003$ ).

**CONCLUSION:** BIS plus split 2-L PEG-CS was as effective as but better tolerated and accepted than split 4-L PEG for screening colonoscopy. This new procedure may increase the positive attitude and participation to colorectal cancer screening colonoscopy.

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**Key words:** Colonoscopy; Colon cleansing; Bowel preparations; Polyethylene glycol; Simethicone; Bisacodyl

**Core tip:** Colorectal cancer ranks as the most common newly-diagnosed cancer in Europe and the second most common cause of cancer death in Europe. A new colon cleansing procedure based on bisacodyl plus polyethylene glycol (PEG) with citrates and simethicone administered as split dose has the same efficacy but superior tolerability and acceptance to split conventional 4-L PEG. This new procedure may increase the positive attitude and participation to colorectal cancer screening colonoscopy.

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## INTRODUCTION

Colorectal cancer (CRC) ranks as the most common newly-diagnosed cancer in Europe and the second most common cause of cancer death in Europe<sup>[1]</sup>. Screening for early detection and removal of premalignant adenomas or localized cancer is crucial to reduce morbidity and mortality associated with CRC<sup>[2,3]</sup>. Colonoscopy is the current gold standard when non-invasive methods are positive (*i.e.*, faecal occult blood test, FOBT) in colorectal cancer population screening programs (> 50 years in Italy) and is also recommended and used as a primary screening modality<sup>[4,5]</sup>. The success of colonoscopy is largely dependent on the level of bowel cleansing<sup>[6]</sup>. Adequate visualization of the colonic mucosa requires a clean colon with no solid or residual brown liquid that could mask a potential lesion. It has been demonstrated that inadequate bowel preparation is associated with lower adenoma detection rates, incomplete colonoscopy or more technically difficult procedure<sup>[7-10]</sup>. A major concern is that detection of lesions in the right colon can be missed due to inadequate bowel preparation<sup>[11]</sup>. The quality of bowel preparation depends on the compliance of the patient, the type of bowel preparation and the timing of ingestion<sup>[12]</sup>.

Polyethylene glycol (PEG) solutions are widely used as they are safe and effective. However the large volume (4 L) to be taken may be a considerable burden for the patient. In clinical practice the reduced tolerability due to large volume and salty taste of PEG solutions may lead to low adherence to the instructions by patients, they drink less than the correct amount with the result of sub-optimal efficacy<sup>[13]</sup>.

Different low-volume formulations have been used such as sodium phosphate and magnesium citrates. They appear to be better tolerated but these solutions should be used with caution in frail patients or patients with renal failure as they can induce dehydration or electrolyte imbalance<sup>[11]</sup>. A more recent option is the addition of ascorbates to the PEG solution or the use of bisacodyl (BIS) for low-volume bowel preparation<sup>[14,15]</sup>.

Also timing and dose administration improve the overall performance and acceptance of bowel preparation<sup>[16,17]</sup>. A split dose regimen, in which the first half dose is taken on the day before and the second half dose on the day of procedure have been shown to be more effective for colon cleansing than single full dose administration on the day before. The split dosing rule appears to be valid for any type of bowel preparation<sup>[18-21]</sup>. A new iso-osmotic sulphate-free PEG electrolyte preparation with citrate and simethicone (PEG-CS) is commercially available to be used with BIS tablets to achieve optimal colon cleansing with threefold mechanism of action<sup>[22,23]</sup>. BIS has a stimulant effect on the colonic motility, while PEG and citrates act as osmotic agents and simethicone favors the foam coalescence improving mucosal visibility<sup>[24-27]</sup>. We therefore compared the efficacy, tolerability and compliance of the new low volume procedure with BIS plus PEG-CS solution versus standard 4-L PEG in

patients undergoing screening colonoscopy.

## MATERIALS AND METHODS

### Subjects selection

Eligible subjects were those referred to colonoscopy as a second level examination following positive FOBT or as a follow-up for adenomatous polyps in the CRC screening promoted by the Veneto region, from December 2009 to January 2011.

Subjects were excluded if they had a history of hypersensitivity to PEG or any other ingredient of products used in the study and other labeled contraindications of the commercially available products.

### Ethical considerations

This study was conducted according to the principles of the Declaration of Helsinki after obtaining approval from the Institutional Review Board. Written informed consent was obtained from each subject.

### Study preparations

All patients were instructed to follow a low-fiber diet for the three days preceding colonoscopy and to drink only clear fluids after starting the bowel preparation.

PEG-CS is an iso-osmotic low volume sulphate-free bowel preparation consisting of PEG 4000, citric acid, sodium citrate, sodium chloride, potassium chloride, simethicone and flavoring agents supplied in four 64.5 g sachets (Lovol-esse, Promefarm). The powder for oral solution must be dissolved in 500 mL of water. This product is combined with BIS 5-mg tablets (Lovoldyl, Promefarm) for full bowel preparation before colonoscopy. In this study, a split dose regimen was used: BIS 15 mg at 16:00 and PEG-CS 1 L at 19:00 the day before. On the day of colonoscopy, PEG-CS 1 L at 7:00 for colonoscopy after 11:00.

The reference preparation was standard PEG electrolyte solution (Isocolan, Bracco), given as split dosing, *i.e.*, 3 L at 17:00 and 1 L at 7:00 for colonoscopy after 11:00 (Table 1).

### Study design

This was a randomised comparative investigator-blind study including consecutive outpatients undergoing screening or follow-up colonoscopy at the Department of Gastroenterology and Digestive Endoscopy at Santa Maria del Prato Hospital in Feltre (Belluno, Italy).

At the time of registration, subjects were randomly allocated in a 1:1 ratio to receive PEG-CS plus BIS or the standard PEG 4-L. Randomization was computer-generated; eligible patients were sequentially numbered and received the corresponding preparation by a nurse in order to ensure adequate concealment.

Study medications were supplied to subjects using the commercially available preparations. Study investigators were kept blinded to study medications and subjects were asked not to reveal the preparation used.

**Table 1** Characteristics of the two polyethylene glycol bowel preparations

	PEG-CS	4-L PEG
Active ingredients	Bisacodyl, PEG, citrates, simethicone	PEG, sodium sulphate
Product description	4 sachets; each containing PEG 4000 60.7 g, sodium citrate 1.066 g, citric acid 1.25 g, simethicone 80 mg	8 sachets; each containing PEG 4000 29.5 g and sodium sulphate 2.843 g
Total volume	2-L	4-L
Electrolytes	Sodium chloride, potassium chloride	Sodium bicarbonate, sodium chloride, potassium chloride
Osmolality (mOsmol/kg)	293	288
Mixed with	Water	Water
Diet prior to colonoscopy	Clear liquid after starting solution intake	Clear liquid after starting solution intake
Timing of intake	1-L of solution at 19:00 the day prior to procedure 1-L of solution at 7:00 the day of the exam	3-L of solution at 17:00 the day prior to procedure 1-L of solution at 7:00 the day of the exam
Additional agents	15 mg bisacodyl (3 tablets) at 16.00 the day prior to procedure	

PEG: Polyethylene glycol; PEG-CS: PEG with citrates and simethicone.

Subjects visited the departments on 2 occasions: enrolment (randomization) and on the day of colonoscopy.

Medical history including concomitant medications, physical examination and vital signs were taken at baseline and on the day of colonoscopy. Subjects received oral and written instructions on the use of the bowel preparation including dietary advice consisting of a 3-d low-fibre diet followed by clear liquids on the day before colonoscopy.

Safety evaluation was based on reporting of adverse events and adverse drug reactions using a standard questionnaire during the visit before colonoscopy.

On the day of colonoscopy the patients were asked to fill a further questionnaire to provide information about whether or not they experienced gastrointestinal (GI) symptoms such as nausea, bloating, and abdominal discomfort, the amount of solution actually taken, difficulty to complete the preparation (3-point scale), taste (3-point scale), willingness to repeat the same preparation in the future (yes or no).

Bowel preparation was evaluated using a 5-point bowel cleansing rating scale for each colonic segment (caecum/ascending colon, transverse, descending and sigmoid colon, rectum)<sup>[12]</sup>. The overall quality of cleansing was based on the assessment of the individual segments using the grade A = all segment clean (*i.e.*, scores of 3 or 4 in all segments); B = brown liquid or removable semi-solid residue (*i.e.*, score of 2) in 1 or more segments; C = semi-solid only partially removable in at least one segment (*i.e.*, score of 1); D = presence of solid stool that can not be removed (*i.e.*, score of 0). In case of D the exam has to be repeated.

Although we used a validated rating scale, the degree

of cleansing remains a matter of personal judgment. In order to minimize this potential issue, four experienced endoscopists (> 5000 procedures in their career) participated in this study after training with the same rating scale by using a set of endophotographs of different segments with various degrees of cleansing.

In the primary analysis, successful bowel cleansing was considered as overall cleansing score equal to A or B. The primary objective of the study was to compare the degree of cleansing of the bowel preparations. It was also assumed that the low volume prep might improve tolerability and acceptability.

### Statistical analysis

The study was designed as a non-inferiority study and sample size was based on an expected rate of successful bowel cleansing of 80% for both groups. Based on practical considerations, the non-inferiority limit was specified as 15%. In order to reach a 80% statistical power with a significant level of 5%, and taking into account a drop-out rate of 10%, no less than 136 patients were needed in each arm.

## RESULTS

Overall, 280 patients were randomly allocated to receive BIS plus PEG-CS ( $n = 140$ ) or standard 4-L PEG ( $n = 140$ ). Colonoscopy data were not available for sixteen patients who did not show up and no information was available with regard to bowel preparation. Therefore 264 patients were included in the analysis of the primary outcome (138 in the PEG-CS and 126 in the 4-L PEG) (Figure 1).

Comparison of demographic characteristics at baseline show no significant differences between the two treatment groups [PEG-CS: male 59.4%; age  $63.6 \pm 7.1$  years; body mass index (BMI)  $27.3 \text{ kg/m}^2$ ; 4-L PEG: male 64.3%; age  $61.3 \pm 7.7$  years; BMI  $27.7 \pm 4.5 \text{ kg/m}^2$ ].

Bowel preparation was successful (grade A + B) for 92.8% of subjects in the PEG-CS group and for 92.1% of subjects in the 4-L PEG (RR = 1.01; 95%CI: 0.94-1.08) (Table 2). There was no statistical difference with regard to the primary outcome of the study also for the grade A alone between the two groups. Bowel cleansing scores according to colonic segment are shown in Table 3. The rates of excellent score were higher for 4-L PEG in the caecum/ascending colon ( $P < 0.02$ ) and in the sigmoid colon ( $P < 0.02$ ) compared with PEG-CS.

A numerically higher number of polyps was observed both in the right and left colon for PEG-CS than 4-L PEG though no statistical difference was found between groups. No adverse events were reported in the study.

The rate of patients with bloating or any GI volume-related symptoms was significantly lower following 2-L PEG-CS + BIS than 4-L PEG (Table 2).

A greater rate of patients in the PEG-CS + BIS had no difficulty and/or were willing to repeat the same preparation than split-dose 4-L PEG. Subjects in the PEG-CS group rated the prep as good or satisfactory in 90.6% as

**Table 2 Primary efficacy and other endpoints *n* (%)**

Outcome	BIS + 2-L PEG-CS ( <i>n</i> = 138)	4-L PEG ( <i>n</i> = 126)	<i>P</i> value
Bowel cleansing			
A (all segments as excellent or good)	107 (77.5)	105 (83.3)	NS
B (at least one segment as fair)	21 (15.2)	11 (8.7)	
C (at least one segment as poor)	7 (5.1)	8 (6.3)	
D (exam not completed)	3 (2.2)	2 (1.6)	
Successful (A + B)	128 (92.8)	116 (92.1)	NS
Unsuccessful (C + D)	10 (7.2)	10 (7.9)	
Tolerability			
Nausea	27 (19.6)	26 (20.6)	0.575
Bloating	11 (8.0)	33 (26.2)	< 0.001
Abdominal discomfort	13 (9.4)	5 (4.0)	0.079
Overall (any of previous ones)	45 (32.6)	57 (45.2)	0.035
Acceptance			
Good	70 (50.7)	30 (23.8)	< 0.001
Satisfactory	55 (39.9)	67 (53.2)	
Not acceptable	13 (9.4)	22 (23.0)	
Compliance			
100% of solution drunk	134 (97.1)	110 (87.3)	0.010
75% of solution drunk	4 (2.9)	15 (11.9)	
50% or less of solution drunk	0 (0.0)	1 (0.8)	

PEG: Polyethylene glycol; PEG-CS: PEG with citrates and simethicone; BIS: Bisacodyl.

compared to 77% in the 4-L PEG,  $P < 0.01$ ) (Table 2).

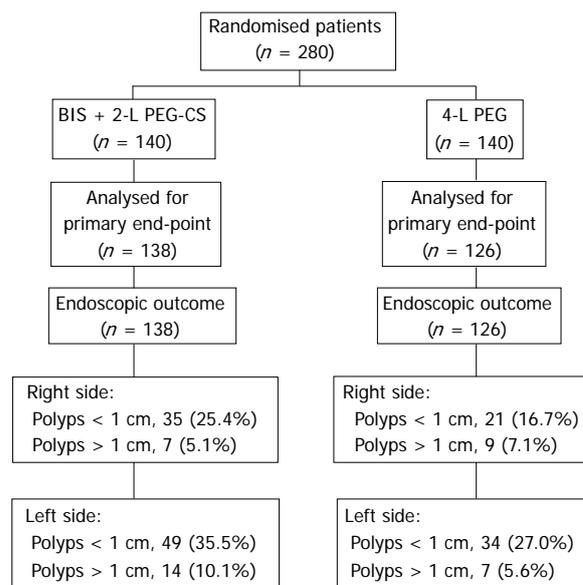
Subjects receiving PEG-CS stated they fully adhered to instructions drinking all the 2-L solution in 97.1% compared with 87.3% in the 4-L PEG ( $P < 0.01$ ) (Table 2).

## DISCUSSION

This study shows that a new isosmotic 2-L PEG formulation with citrate and simethicone plus BIS tablets was as effective as split 4-L PEG and electrolytes for bowel cleansing in subjects undergoing FOBT-positive screening colonoscopy. The low-volume formulation was associated with better tolerability, acceptability and compliance to instructions received for bowel preparation. Complete bowel preparation is an important component to ensure high quality in colonoscopy and minimize the risk of missing polyps and lesions<sup>[9,28]</sup>. Current PEG-based bowel preparations are safe but inadequate bowel preparation is common with about 25% of patients with inadequate colon cleansing and about 4%-5% of patients who have to repeat colonoscopy<sup>[7,8]</sup>. However the large volume of fluids, salty taste and difficulty to complete the preparation remain a deterrent for patients undergoing colonoscopy and to a greater extent for asymptomatic subjects invited to a screening CRC program.

Efforts have been made by the pharmaceutical industry to satisfy the need to reduce the burden and make PEG bowel preparation easier and more acceptable for patients without changing the level of efficacy and safety.

The new procedure based on BIS tablets and split PEG-CS appears to be a valuable option. In our study it provided a level of overall cleansing similar to split 4-L



**Figure 1** Enrolment, randomization and endoscopy outcome. PEG: Polyethylene glycol; PEG-CS: PEG with citrates and simethicone; BIS: Bisacodyl.

PEG. Previous studies have shown that BIS tablets plus 2-L PEG-CS given the day before is equally as effective and safe as 4-L PEG for bowel cleansing before colonoscopy<sup>[22,23]</sup>. There are important differences with earlier trials, regarding the PEG-formulation and the dose regimen.

First, the formulation of the low-volume PEG solution is different from the standard 4-L PEG. PEG-CS is sulphate-free, contains new active ingredients (citric acid, sodium citrate and simethicone) and a higher concentration of PEG per litre of reconstituted solution than the traditional PEG formulation. From our data, there is no clue to determine the relative contribution of BIS tablets or any other ingredient of the bowel preparation for bowel cleansing.

Second, this study compared the split dosing regimen for both the low-volume and reference bowel preparation. According to ACG guidelines, split dose bowel preparation enhances the quality of bowel preparation and therefore is now recommended for all patients undergoing screening or surveillance colonoscopy<sup>[2]</sup>. When a part of the bowel preparation is taken within 4-8 h of colonoscopy, there is a better cleansing of caecum and the ascending colon compared with traditional dosing schedule of the day before. With such regimen the long interval of > 12 h between bowel preparation and colonoscopy allows the flow of intestinal secretion across the ileo-caecum valve and yellow fluid cover mucosa of the right colon. For the full-dose, we have used an unequal split (PEG 3-L the day before and 1-L the day before the same day) which have been shown to be effective but more feasible as it allows to perform colonoscopy shortly in the morning<sup>[29]</sup>.

In our study BIS tablets were taken in the afternoon before, 1 L of PEG-CS was taken in the evening and

**Table 3** Quality of cleansing for each colonic segment

Score	Caecum/ascending colon		Transverse colon		Descending colon		Sigmoid colon		Rectum	
	BIS + 2-L PEG- CS (n = 138)	4-L PEG (n = 126)	BIS + 2-L PEG- CS (n = 138)	4-L PEG (n = 126)	BIS + 2-L PEG- CS (n = 138)	4-L PEG (n = 126)	BIS + 2-L PEG- CS (n = 138)	4-L PEG (n = 126)	BIS + 2-L PEG- CS (n = 138)	4-L PEG (n = 126)
Excellent	37.00%	52.40%	52.20%	57.90%	51.40%	59.50%	45.70%	60.30%	40.60%	51.60%
Good	50.70%	37.30%	38.40%	34.90%	37.00%	31.00%	39.90%	28.60%	43.50%	39.70%
Fair	7.20%	4.00%	5.10%	2.40%	6.50%	5.60%	9.40%	5.60%	12.30%	4.00%
Poor	2.90%	4.80%	2.20%	3.20%	5.10%	3.20%	5.10%	4.80%	3.60%	4.00%
Missing	2.20%	1.60%	2.20%	1.60%	0.00%	0.80%	0.00%	0.80%	0.00%	0.80%

PEG: Polyethylene glycol; PEG-CS: PEG with citrates and simethicone; BIS: Bisacodyl.

1 L in the morning about 4 h before colonoscopy. The important finding of this study is that for the first time a 2-L PEG preparation administered as a split-dose was shown to be as globally effective as 4-L PEG. Although this finding needs to be confirmed in future trials, a better cleansing in the right colon may favor the detection of small or flat lesions which are more likely to remain undetected compared to other sites of the colon<sup>9,10</sup>.

Any new low-volume bowel preparations should also be evaluated on the grounds of safety, tolerability, acceptance and compliance.

PEG-CS is an osmotically balanced PEG solution and therefore it is less likely to induce electrolyte imbalance as compared to bowel preparations based on sodium phosphate, magnesium phosphate or hyperosmotic PEG solutions. Based on vital signs, haemodynamic data and lack of extra-intestinal adverse events, no safety issue was identified in this study for both bowel preparations.

Similarly to standard 4-L PEG no issues of safety, in particular electrolyte imbalance and dehydration are associated with the new formulation.

The new formulation was significantly better tolerated and accepted by patients. A reduced rate of bloating and cumulative volume-related GI symptoms were observed in BIS plus PEG-CS than in the reference group. This is not surprising as a much lower amount of non-absorbable fluid must be taken for each session with PEG-CS (1-L only) than traditional PEG-formulation. The new formulation was also shown to be less difficult to complete, of pleasant taste and patients were more willing to repeat it for future examination. Although split-dosing itself may increase acceptance of bowel preparation, the features of this low-volume colon cleansing procedure improved acceptance, compliance and adherence to bowel preparation instructions.

This low-volume bowel preparation may be a first option for patients who poorly tolerated and accepted large volume bowel preparations for colonoscopy. Better tolerability, acceptance and compliance to bowel preparation may increase the attitude and uptake to screening colonoscopy. This requires a high level of quality as the objective of the examination is to detect even small but potentially dangerous lesions which may progress to cancer.

This single-centre study was carried out in a homogenous group of patients undergoing screening colonos-

copy. It should be noted that the high rate of adequate bowel cleansing in both groups is largely due to the fact the sample group was made of motivated subjects aged between 50 and 69 years who came for a second level examination after being found positive to occult blood. Our finding may have important implication for the general population or elderly people who may have lower levels of motivation to undergo colonoscopy also due to bowel preparation.

There are concerns about ischemic colitis related to BIS. To date, no reports of ischemic colitis were observed in the clinical trials reviewed and in the post-marketing pharmacovigilance according to the manufacturer. A causal relationship between use of BIS for colon cleansing and ischemic colitis remains to be established.

Over the last years the pharmaceutical industry has tried to offer bowel preparations which are better accepted without compromising efficacy and safety. Although further evidence is needed, it seems from our study that an important advance toward optimal and easy bowel preparation has been made.

In conclusion, this study evaluated a new low-volume bowel preparation for FOBt-positive screening colonoscopy. BIS plus PEG-CS was as effective but better tolerated and accepted than split 4-L PEG. Bowel preparation before colonoscopy has for a long time been an issue for our patients. Now progress has been made towards better tolerability, acceptance and compliance of bowel preparation. Reducing the burden for healthy subjects may improve their attitude and maximize the benefits of screening colonoscopy.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Quality of bowel preparation is essential to identify lesions in the colon. The running time between the last dose of bowel preparation and the exam has been shown to play a key role toward the ideal bowel cleansing.

### Research frontiers

Split dose regimen with a fraction of bowel preparation taken the day of the exam may be an effective approach in clinical practice.

### Innovations and breakthroughs

A new colon cleansing procedure based on bisacodyl (BIS) plus polyethylene glycol (PEG) with citrates and simethicone (PEG-CS) administered as split dose has the same efficacy but superior tolerability and acceptance to split conventional 4-L PEG.

### Applications

The study results suggest that the split dose of the low volume PEG-CS after BIS increases the patient attitude and acceptance to colorectal cancer screening colonoscopy.

### Peer review

This is a well designed and written study that adds to the authors' understanding of optimal bowel preparation regimens.

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## Fucoidan enhances intestinal barrier function by upregulating the expression of claudin-1

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### Abstract

**AIM:** To evaluate the protective effects of fucoidan on oxidative stress-induced barrier disruption in human intestinal epithelial cells.

**METHODS:** In Caco-2 cell monolayer models, the disruption of barrier function by oxidative stress is mediated by H<sub>2</sub>O<sub>2</sub>. The integrity of polarized Caco-2 cell monolayers was determined by measuring the transepithelial resistance (TER) and permeability was estimated by measuring the paracellular transport of FITC-labeled 4-kDa dextran (FD4). The protective effects of fucoidan on epithelial barrier functions on polarized Caco-2 cell monolayers were evaluated by TER and FD4 flux. The

expression of tight junction (TJ) proteins was assessed using reverse-transcription polymerase chain reaction (RT-PCR) and immunofluorescence staining.

**RESULTS:** Without H<sub>2</sub>O<sub>2</sub> treatment, fucoidan significantly increased the TER compared to control ( $P < 0.05$ ), indicating a direct enhancement of intestinal epithelial barrier function. Next, H<sub>2</sub>O<sub>2</sub> disrupted the epithelial barrier function in a time-dependent manner. Fucoidan prevented the H<sub>2</sub>O<sub>2</sub>-induced destruction in a dose-dependent manner. Fucoidan significantly decreased H<sub>2</sub>O<sub>2</sub>-induced FD4 flux ( $P < 0.01$ ), indicating the prevention of disruption in paracellular permeability. RT-PCR showed that Caco-2 cells endogenously expressed claudin-1 and -2, and occludin and that H<sub>2</sub>O<sub>2</sub> reduced the mRNA expression of these TJ proteins. Treatment with fucoidan attenuated the reduction in the expressions of claudin-1 and claudin-2 but not occludin. Immunofluorescence staining revealed that the expression of claudin-1 was intact and high on the cell surface. H<sub>2</sub>O<sub>2</sub> disrupted the integrity of claudin-1. Treatment with fucoidan dramatically attenuated the expression of claudin-1.

**CONCLUSION:** Fucoidan enhanced intestinal epithelial barrier function by upregulating the expression of claudin-1. Thus, fucoidan may be an appropriate therapy for the treatment of inflammatory bowel diseases.

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**Key words:** Fucoidan; Tight junction; Intestinal epithelial cells; Oxidative stress; Inflammatory bowel diseases

**Core tip:** The oxidative stress-induced disruption of the intestinal epithelial cells and subsequent increased paracellular permeability are critically important in the pathogenesis of inflammatory bowel diseases (IBD). A growing body of experimental evidence indicates that fucoidan, a dietary substance of fucose-enriched

sulfated polysaccharides, display a wide variety of pharmacological anti-inflammatory activities. This study demonstrates that fucoidan protected the epithelial barrier function from oxidative injury of the tight junction as well as barrier disruption by upregulating the expression of claudin-1. Thus, fucoidan may be an appropriate therapy for the treatment of IBD.

Iraha A, Chinen H, Hokama A, Yonashiro T, Kinjo T, Kishimoto K, Nakamoto M, Hirata T, Kinjo N, Higa F, Tateyama M, Kinjo F, Fujita J. Fucoidan enhances intestinal barrier function by up-regulating the expression of claudin-1. *World J Gastroenterol* 2013; 19(33): 5500-5507 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i33/5500.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5500>

## INTRODUCTION

Although the gastrointestinal (GI) tract is constantly exposed to bacterial microflora, an excess immune response against the bacterial microflora does not occur in the normal state, as a result of some type of immunological tolerance underlying the GI immune system. However, disruption of this immunological tolerance against intestinal microbial antigens may cause abnormal intestinal inflammation and the development of chronic inflammatory diseases, such as inflammatory bowel diseases (IBD)<sup>[1,2]</sup>. IBD can be classified into two distinct diseases, ulcerative colitis (UC) and Crohn's disease (CD). Although the precise etiology of these diseases remains unclear, several reports have indicated that intestinal microflora is responsible for the pathogenesis of both UC and CD<sup>[3,4]</sup>. Intestinal epithelial cells (IEC) play a role as the first line of defense and act as a functional barrier. IECs separate the host's internal milieu from the external environment. In addition to functioning as a barrier, it has become evident that IECs also play an important role in the maintenance of immune homeostasis<sup>[5]</sup>. IECs produce anti-microbial peptides, such as defensins, and protect the host from the attachment of luminal bacteria<sup>[6]</sup>. Not only do IECs function in a direct bacteriocidal role, but IEC-derived factors can also promote the differentiation of anti-inflammatory types of dendritic cells and macrophages to induce mucosal tolerance against luminal bacteria<sup>[7,8]</sup>. Furthermore, in intestinal inflammation, IECs can produce several chemokines and pro-inflammatory cytokines in response to luminal bacteria to induce the migration of granulocytes, lymphocytes, and dendritic cells, resulting in the induction of host immunity. Thus, IECs function as a defensive frontline of host mucosal immunity. Accordingly, direct epithelial cell damage, induced by mucosal irritants or cytotoxic agents, results in a marked loss of barrier function<sup>[9]</sup>. The epithelial barrier consists of several essential elements, including an intact epithelial monolayer and the tight junction (TJ). The TJ consists of four integral membrane proteins: occludins,

claudins, tricellulin and the junctional adhesion molecule. A large body of evidence indicates that disruption of the TJ and increased paracellular permeability are critically important in the pathogenesis of IBD<sup>[10]</sup>. The oxidative stress-induced opening of the TJ barrier is an important mechanism contributing to the TJ barrier defect present in IBD<sup>[11]</sup>.

Caco-2, a human intestinal epithelial cell line, is the most well studied cell line for investigations of *in vivo* intestinal epithelial barrier integrity and function<sup>[12]</sup>. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a highly toxic oxidizing agent, is constantly generated within intestinal epithelial cells and must quickly be detoxified by antioxidant enzymes<sup>[13]</sup>. It has been established that H<sub>2</sub>O<sub>2</sub> is involved in oxidative stress-induced cell injury and disrupts intestinal epithelial barrier function, thus leading to enhanced paracellular permeability and the promotion of marked changes in the expression and/or localization of a number of TJ proteins, including claudins and occludins. In Caco-2 cell monolayer models, the disruption of barrier function by oxidative stress is mediated by H<sub>2</sub>O<sub>2</sub><sup>[13]</sup>.

Fucoidan, a dietary substance, represents a class of fucose-enriched sulfated polysaccharides found in the extracellular matrix of brown algae. A growing body of experimental evidence indicates that fucoidans display a wide variety of pharmacological activities, including anti-inflammatory, anti-angiogenic, anti-coagulant, and anti-adhesive effects, in experimental models<sup>[14-16]</sup>. Thus, great interest has been generated in investigating the potential pharmacological effects of fucoidan on H<sub>2</sub>O<sub>2</sub>-induced TJ destruction in IECs.

In this study, we examined the protective effect of fucoidan on H<sub>2</sub>O<sub>2</sub>-induced TJ destruction in human IECs, which may provide a novel approach for the treatment of IBD.

## MATERIALS AND METHODS

### Materials

The brown algae *Cladosiphon okamuranus Tokida* was cultivated in Okinawa, Japan. Purified fucoidan derived from *C. o. Tokida* was provided by Uruma Bio Co. Ltd., (Okinawa, Japan). Fucoidan was dissolved in Dulbecco's Vogt modified Eagle's media (DMEM) (Sigma-Aldrich Co., St. Louis, MO).

### Cell culture

A human intestinal epithelial cell line, Caco-2 cells (RBRC-RCB0988 RIKEN Bio Resource Center, Ibaraki, Japan), were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS (Nichirei Biosciences Inc., Tokyo, Japan), 100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies Gibco, France), and 10 ml GlutaMAX™ (100 ×) (Life Technologies Gibco, France). The cell cultures were incubated on collagen-coated tissue culture plates Transwell® (Corning, New York, NY) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

### Measurement of transepithelial resistance

The integrity of polarized Caco-2 cell monolayers was determined by measuring the transepithelial resistance (TER), which reflects the tightness of the TJ between epithelial cells<sup>[17,18]</sup>. The TER was measured in  $\Omega\text{cm}^2$  using a Millicell ERS-2 Epithelial Volt-Ohm Meter (Millipore, Bedford, MA). Caco-2 cells were cultured on 24 mm Transwell<sup>®</sup> polycarbonate inserts (0.4  $\mu\text{mol/L}$  pore size) for 14 to 21 d. To examine the direct effect of fucoidan on well-polarized Caco-2 cell monolayers, confluent polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) in apical medium for 24 h. To evaluate the protective effects of fucoidan on epithelial cell injury, serial doses of fucoidan (0, 0.1, 1.0, or 2.5 mg/mL) were added to the apical medium 30 min prior to the administration of  $\text{H}_2\text{O}_2$  (500  $\mu\text{mol/L}$ ) to the basolateral side of the Transwell<sup>®</sup>. Changes in the TER during the experimental periods were calculated as the percentage of the corresponding basal values. TER of unseeded inserts was subtracted.

### Macromolecular permeability (FITC-dextran flux assay)

Permeability was estimated by measuring the paracellular transport of FITC-labeled 4-kDa dextran (FD4) (Molecular Probes, Netherland). Once the cells were grown to confluence ( $\text{TER} > 350 \Omega\text{cm}^2$ ), sterilized FD4 was added into the apical well at 1 mg/mL.  $\text{H}_2\text{O}_2$  (500  $\mu\text{mol/L}$ ) was administered to the basolateral side of the Transwell<sup>®</sup>. Fucoidan (2.5 mg/mL) was added to the apical medium 30 min prior to  $\text{H}_2\text{O}_2$  administration. After 6 h of incubation, the basolateral medium was collected, and the fluorescence was measured using a fluorescence spectrometer at an excitation of 485 nm and emission of 535 nm. The permeability was expressed as the percentage of fluorescence of the  $\text{H}_2\text{O}_2$ -treated group. Flux of unseeded inserts was subtracted.

### Analysis of tight junction protein mRNA expression using reverse-transcription polymerase chain reaction

Caco-2 cells were cultured for 14 to 21 d. Once grown to confluence ( $\text{TER} > 350 \Omega\text{cm}^2$ ),  $\text{H}_2\text{O}_2$  (500  $\mu\text{mol/L}$ ) was administered to the basolateral side of the Transwell<sup>®</sup>. Fucoidan (2.5 mg/mL) was added to the apical medium 30 min prior to  $\text{H}_2\text{O}_2$  administration. After 24 h of incubation, the cells were harvested, and total RNA was isolated using the RNeasy Mini kit (Qiagen, KJ Venlo, the Netherlands). Isolated RNA was treated with RNase-free DNase I (Qiagen) to prevent any carry-over of genomic DNA. The cDNA was synthesized from 2  $\mu\text{g}$  of total RNA with Quantitect reverse transcriptase (Qiagen). Reverse-transcription polymerase chain reaction (RT-PCR) was performed using a PCR master mix (Takara Biosystems, Foster City, CA). Primers were listed 5'-3' as follows: Claudin-1: F, GCG CGA TAT TTC TTC TTG CAG G; R, TTC GTA CCT GGC ATT GAC TGG. Claudin-2: F, CTC CCT GGC CTG CAT TAT CTC; R, ACC TGC TAC CGC CAC TCT GT. Occludin: F, TCA

GGG AAT ATC CAC CTA TCA CTT CAG; R, CAT CAG CAG CAG CCA TGT ACT CTT CAC.

### Immunofluorescence staining of TJ proteins

Caco-2 cells were cultured for 14 to 21 d on a Lab-Tek chamber plate (Corning).  $\text{H}_2\text{O}_2$  (500  $\mu\text{mol/L}$ ) was administered to the basolateral side of the Transwell<sup>®</sup>. Fucoidan (2.5 mg/mL) was added to the apical medium 30 min prior to  $\text{H}_2\text{O}_2$  treatment. After 6 h of incubation, the cells were washed twice with cold PBS and fixed with cold acetone (Wako Pure Chemical Industries, Osaka, Japan) for 10 min. The cells were then removed from the Transwell<sup>®</sup> and mounted on slides. Next, the cells were incubated with mouse anti-human claudin-1 (Zymed Laboratories, San Francisco, CA) at 4 °C overnight. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Netherland) then subsequently washed in PBS. The immunofluorescence was examined and imaged using fluorescence microscopy (Nikon Eclipse 80i).

### Statistical analysis

Statistical analysis was performed using the GraphPad Prism software program, version 4.0 (GraphPad Software Inc., San Diego, CA). Differences with  $P < 0.05$  were considered significant. All of the data were expressed as the means  $\pm$  SEM.

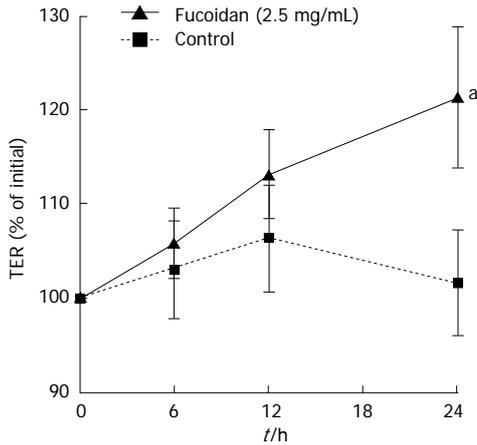
## RESULTS

### Fucoidan directly enhanced intestinal epithelial barrier function

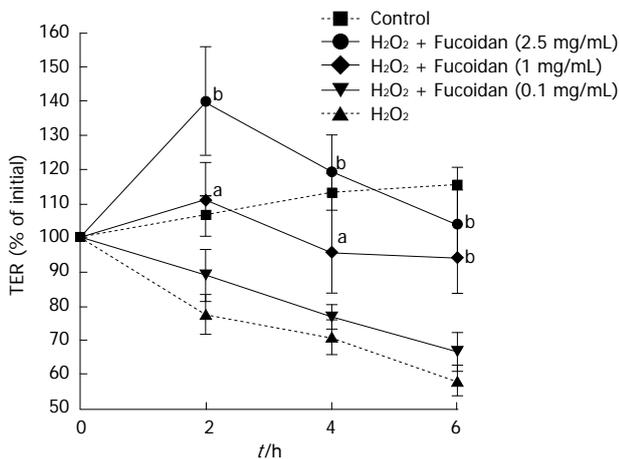
First, we determined the effect of fucoidan on the protective functions of Caco-2 cell monolayers. To determine whether fucoidan directly induced the increase in epithelial resistance or TER was upregulated by the promotion of epithelial cell proliferation, we examined the effect of fucoidan on well-polarized Caco-2 cell monolayers. Completely polarized Caco-2 cell monolayers showed approximately 600  $\Omega\text{cm}^2$  TER. Because polarized Caco-2 cells could not further proliferate, the direct effect of fucoidan on intestinal epithelial barrier functions could be examined. Interestingly, fucoidan significantly increased the TER ( $P < 0.05$  compared with control), indicating an enhancement of intestinal epithelial barrier function (Figure 1).

### Fucoidan prevented $\text{H}_2\text{O}_2$ -induced destruction of intestinal epithelial barrier function in a dose-dependent manner

Next, we focused on the preventive effects of fucoidan on epithelial cell injury. To assess the effect of fucoidan on intestinal epithelial barrier function, completely polarized Caco-2 cell monolayers were injured using  $\text{H}_2\text{O}_2$  (500  $\mu\text{mol/L}$ ).  $\text{H}_2\text{O}_2$  was added into the lower well of the Transwell<sup>®</sup> and changes in intestinal epithelial barrier function were monitored by measuring the TER. As shown in Figure 2,  $\text{H}_2\text{O}_2$  disrupted the epithelial barrier function

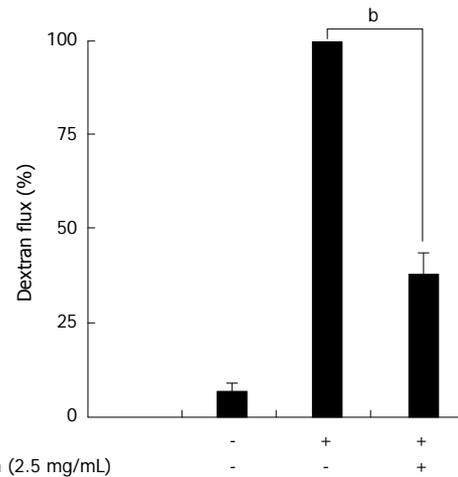


**Figure 1** Fucoidan directly enhanced intestinal epithelial barrier function. Polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) for 24 h. Changes in intestinal epithelial barrier function were monitored by measuring the trans-epithelial resistance (TER). The data are expressed as the means  $\pm$  SEM of 5 independent experiments. <sup>a</sup> $P < 0.05$  compared with control (Student's *t* test).



**Figure 2** Fucoidan prevented H<sub>2</sub>O<sub>2</sub>-induced destruction of intestinal epithelial barrier function in a dose-dependent manner. Polarized Caco-2 cell monolayers were injured by H<sub>2</sub>O<sub>2</sub> (500  $\mu$ mol/L) on the apical side of Caco-2 cell monolayers. Fucoidan was added into the basolateral side 30 min prior to H<sub>2</sub>O<sub>2</sub> stimulation and cultured for 6 h. Changes in intestinal epithelial barrier function were monitored by measuring the trans-epithelial resistance (TER). The data are expressed as the means  $\pm$  SEM of 5 independent experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  compared with cells exposed to H<sub>2</sub>O<sub>2</sub> alone at respective time point (Tukey's multiple comparison test).

in a time-dependent manner. In contrast, treatment with fucoidan prevented H<sub>2</sub>O<sub>2</sub>-induced intestinal epithelial injury at an early time point ( $P < 0.05$ ,  $P < 0.01$  compared with cells exposed to H<sub>2</sub>O<sub>2</sub> alone at respective time point). However, low dose (0.1 mg/mL) of fucoidan did not protect the intestinal epithelium against H<sub>2</sub>O<sub>2</sub> injury after 4 h of exposure; however, high doses (1 and 2.5 mg/mL) of fucoidan prevented the disruption of the epithelial barrier to some extent even at the late phase. Thus, fucoidan prevented H<sub>2</sub>O<sub>2</sub>-induced destruction of the intestinal epithelial barrier in a dose-dependent manner.



**Figure 3** Fucoidan prevented H<sub>2</sub>O<sub>2</sub>-induced increases in paracellular permeability. First, 0.5 mg/mL 4-kDa FITC-labeled dextrans (FD4) were added into the apical well and cultured for 6 h with or without H<sub>2</sub>O<sub>2</sub> (500  $\mu$ mol/L) and/or fucoidan (2.5 mg/mL). After 6 h of incubation, the basal medium was collected, and the fluorescence was measured as fluxed-FD4. H<sub>2</sub>O<sub>2</sub>-induced FD4 flux was considered 100%. The data are expressed as the means  $\pm$  SEM of 5 independent experiments. <sup>b</sup> $P < 0.01$  (Student's *t* test).

### Fucoidan prevented H<sub>2</sub>O<sub>2</sub>-induced increases in paracellular permeability

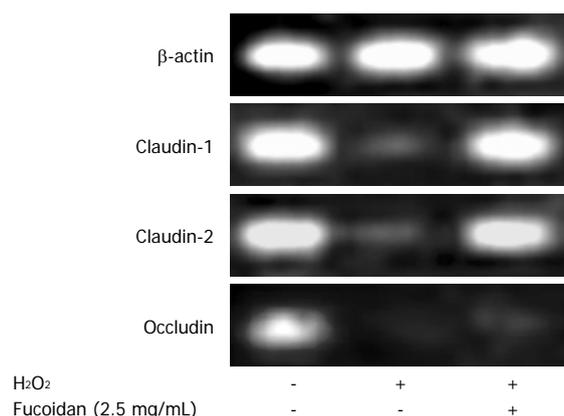
Next, we examined whether H<sub>2</sub>O<sub>2</sub> increased the paracellular permeability of Caco-2 cell monolayers following epithelial injury and whether fucoidan could prevent this effect. For this experiment, an FD4 flux assay was performed. H<sub>2</sub>O<sub>2</sub> markedly increased FD4 flux into the lower well (Figure 3). As expected, pretreatment with fucoidan 30 min prior to H<sub>2</sub>O<sub>2</sub> administration significantly suppressed the increase in FD4 flux into the lower well across the Caco-2 cell monolayers ( $P < 0.01$ ) (Figure 3). These results suggested that H<sub>2</sub>O<sub>2</sub> functionally injured the Caco-2 cell monolayers and that fucoidan prevented the disruption of intestinal epithelial barrier function.

### Fucoidan promoted intestinal epithelial barrier function via direct upregulation of tight junction proteins in IECs

To determine how fucoidan treatment promotes an increase in intestinal epithelial barrier function, we examined the effect of fucoidan on the mRNA expression of major TJ-associated proteins. As shown in Figure 4, Caco-2 cells endogenously expressed claudin-1 and -2, and occludin. H<sub>2</sub>O<sub>2</sub> reduced the mRNA expression of these proteins. In addition, pretreatment with fucoidan attenuated the reduction in the expressions of claudin-1 and claudin-2 mRNA but not occludin mRNA. These results suggested that fucoidan treatment strongly induced the expression of claudin-1 and -2 that promote intestinal epithelial barrier function.

### Fucoidan prevented H<sub>2</sub>O<sub>2</sub>-induced destruction of the tight junction protein claudin-1

We further examined the effect of fucoidan on the inter-



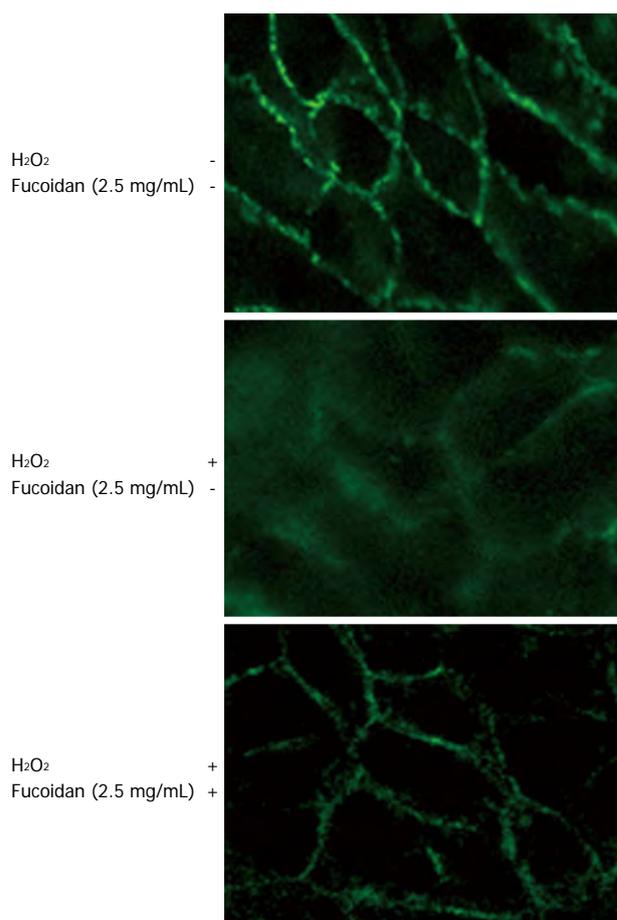
**Figure 4** Fucoidan promoted intestinal epithelial barrier function via direct upregulation of tight junction proteins in intestinal epithelial cells. Polarized Caco-2 cell monolayers injured by H<sub>2</sub>O<sub>2</sub> (500  $\mu$ mol/L) for 24 h with or without pretreatment of fucoidan (2.5 mg/mL) 30 min prior to H<sub>2</sub>O<sub>2</sub> administration. The expression of tight junction proteins, including claudin-1, claudin-2, and occludin, was examined using reverse-transcription polymerase chain reaction. The data shown are representative and are from 1 of the 3 independent experiments.

cellular localization of claudin-1 using immunofluorescence microscopy. We found that the expression of claudin-1 was intact and high on the cell surface in control cells. H<sub>2</sub>O<sub>2</sub> strongly disrupted the integrity of claudin-1, resulting in lower expression. Furthermore, pretreatment with fucoidan dramatically attenuated the H<sub>2</sub>O<sub>2</sub>-induced injury, restoring cell integrity and promoting the expression of claudin-1 (Figure 5).

## DISCUSSION

IBD is associated with an epithelial barrier defect characterized by impaired absorptive function and increased mucosal barrier defects, which are caused by impaired TJ complexity, particularly affecting claudins<sup>[19,21]</sup>. Whereas claudin-1, -3, -4, -5 and -8 demonstrate sealing functions, claudin-2, -10b and -15 act as paracellular channels and promote the charge-selective passage of small ions<sup>[10]</sup>. Recent studies have revealed that the expression of barrier-forming claudin-1 and -4 and occludin are downregulated in the intestinal epithelia of patients with UC<sup>[22]</sup>, and downregulation of claudin-3, -5 and occludin have been observed in CD<sup>[23]</sup>. However, the pore-forming protein claudin-2 is upregulated in both UC and CD, resulting in leaky TJ strands<sup>[22,23]</sup>. Amasheh *et al.*<sup>[24]</sup> recently established an experimental IBD model of native colon *in vitro*, which showed an impairment of epithelial barrier function via downregulation of claudin -1, -5, and -7 after exposure to tumor necrosis factor (TNF)- $\alpha$  and interferon gamma (IFN)- $\gamma$ . Because the present study showed the impaired expression of claudin-1 and occludin by oxidative stress, our model mimicked the intestinal inflammation observed in IBD.

Fucoidans represent an intriguing group of naturally occurring polysaccharides that might have promising therapeutic applications in various clinical situations. Algal



**Figure 5** Fucoidan prevented the H<sub>2</sub>O<sub>2</sub>-induced destruction of tight junction protein claudin-1. Caco-2 cells were grown on a Lab-Tek chamber plate. Polarized Caco-2 monolayers were injured by H<sub>2</sub>O<sub>2</sub> (500  $\mu$ mol/L) for 6 h with or without pretreatment of fucoidan (2.5 mg/mL). Immunofluorescence staining for claudin-1 was evaluated using confocal laser scanning microscopy. The data shown are representative and are from 1 of the 3 independent experiments.

fucoidans are characterized by a wide variety of biological functions and by a highly complex and heterogeneous structure, which varies within algal species. Fucoidans from various algal species might differentially affect inflammation. Although numerous biological activities of fucoidan have attracted attention, only a few studies have examined the pharmacological activity of fucoidan in intestinal inflammation<sup>[25]</sup>. Matsumoto *et al.*<sup>[26]</sup> have shown that the oral administration of fucoidan ameliorated murine chronic colitis by downregulating the synthesis of interleukin-6 (IL-6), a key pro-inflammatory cytokine in IBD, in colonic epithelial cells. They concluded that fucoidan derived from *C. o. Tokida* might be useful as a dietary substance for the treatment of IBD. In addition, Zhang *et al.*<sup>[27]</sup> revealed that intravenous administration of fucoidan reduced colonic mucosal damage and crypt destruction of dextran sodium sulfate-induced murine chronic colitis by reducing colonic myeloperoxidase activity and abolishing TNF- $\alpha$ -induced venular leukocyte rolling and extravascular recruitment. Moreover, Tanoue *et al.*<sup>[28]</sup> established an *in vitro* model of a co-culture system using intestinal epithelial Caco-2 cells and macrophage RAW264.7 cells

to treat intestinal inflammation by fucoidan. They clearly showed that fucoidan suppressed IL-8 gene expression in epithelial cells *via* reduction in TNF- $\alpha$  production from macrophages stimulated with lipopolysaccharide. For gastric inflammation, fucoidan has been found to protect against aspirin-induced gastric ulceration by inhibiting IL-6, TNF- $\alpha$ , and IFN- $\gamma$ <sup>[29,30]</sup>. However, to the best of our knowledge, our study is the first to report that fucoidan protects and strengthens epithelial barrier function, both under physiological and pathological conditions *via* induction of the expression of claudin-1 in human IECs. The mechanisms how fucoidan regulates the TJ proteins in this study are unknown. We next plan to investigate cytokine studies and signaling pathways which may regulate the expression of claudins and occludin by the treatment of fucoidan with a consistent time course experiments.

Pro-inflammatory cytokines, such as TNF- $\alpha$ , IFN- $\gamma$ , and IL-13, affect the expression of TJ proteins in IECs and induce epithelial cell apoptosis, resulting in the disruption of intestinal epithelial barrier function<sup>[22,31,32]</sup>. Because IECs function as a defensive frontline of host mucosal immunity in the intestine, disruption of barrier function of IECs causes an excessive immune response to intestinal bacteria<sup>[5]</sup>. Thus, dysfunction of IECs strongly contributes to the pathogenesis of bacteria-triggered chronic inflammation of the intestine in IBD. However, defects in TJ barrier function are insufficient to cause disease. Increased paracellular permeability can increase mucosal immune activity and enhance disease progression and severity. Thus, restoration of TJ barrier function might be effective, either alone or in combination with other agents, in preventing disease in at-risk individuals or maintaining remission in patients with IBD<sup>[9]</sup>. Although recent advances in anti-TNF- $\alpha$  antibody therapy can dramatically inhibit intestinal inflammation, strengthening the intestinal epithelial barrier is still challenging and has been eagerly investigated. It is well known that zinc, a trace element, assists with the maintenance of intestinal barrier integrity. Glutamine, an essential amino acid, supports recovery from a loss in TER. Moreover, the expression of claudin-1 and occludin proteins were decreased when Caco-2 cells were deprived of glutamine through inhibition of glutamine synthetase<sup>[33]</sup>. Furthermore, a direct influence on TJ protein expression has been observed from several plant components, including the flavonoid quercetin and the isoquinoline alkaloid berberine<sup>[34]</sup>. Quercetin, which is obtained from fruits, enhances barrier function by upregulating claudin-4 expression<sup>[35]</sup>, whereas berberine, a herbal agent, prevented the barrier impairment induced by TNF- $\alpha$  and IFN- $\gamma$ <sup>[36]</sup>. We have demonstrated that fucoidan directly induced the expression of some TJ proteins and might contribute to the enhancement of epithelial barrier functions. Thus, we believe that the activity of fucoidan, which increases the epithelial protective function and promotes epithelial regeneration, might serve as an appropriate therapy for the treatment of IBD.

Although dietary components may regulate TJ permeability by directly targeting the signal transduction

pathways involved in TJ regulation, specific dietary components have been identified that influence cytokine signaling, thereby modifying TJ permeability<sup>[34]</sup>. The intestinal barrier is a complex environment, and the regulation of barrier function cannot be elucidated using *in vitro* models alone. Interactions between dietary components and microbiota are also crucial in the regulation of barrier integrity<sup>[34]</sup>. It is important to consider the interactions between different components of the intestinal barrier when establishing strategies to enhance barrier integrity using dietary compounds. The present study may provide insight for the development of novel agents with low toxicity in the treatment of intestinal inflammation. Because the healing of intestinal inflammation is a complex process involving numerous factors, further work is required to elucidate the therapeutic effect of fucoidan.

## COMMENTS

### Background

The oxidative stress-induced disruption of the intestinal epithelial cells and subsequent increased paracellular permeability are critically important in the pathogenesis of inflammatory bowel diseases (IBD). Although recent advances in anti-tumor necrosis factor- $\alpha$  antibody therapy can dramatically inhibit intestinal inflammation, strengthening the intestinal epithelial barrier is still challenging and has been eagerly investigated.

### Research frontiers

Recent studies have revealed that some dietary components may regulate tight junction (TJ) permeability by directly targeting the signal transduction pathways involved in TJ regulation. A growing body of experimental evidence indicates that fucoidan, a dietary substance of fucose-enriched sulfated polysaccharides with low toxicity, display a wide variety of pharmacological anti-inflammatory activities. However, only a few studies have examined the protective effects of fucoidan for intestinal inflammation.

### Innovations and breakthroughs

The authors investigated the effect of fucoidan on oxidative stress-induced barrier disruption in a Caco-2 cell monolayer model, with an emphasis on the alterations of TJ proteins. This study demonstrates that fucoidan protected the epithelial barrier function from oxidative injury of the TJ as well as barrier disruption by upregulating the expression of claudin-1.

### Applications

Fucoidan may be an appropriate therapy to control the expression of claudin-1 for the treatment of IBD.

### Terminology

Tight junctions: TJ forms a network of close contacts between membranes of adjacent cells. TJ consists of four integral membrane proteins: claudins, occludins, tricellulin and the junctional adhesion molecule. A large body of evidence indicates that disruption of the TJ and increased paracellular permeability are critically important in the pathogenesis of IBD. Fucoidan: fucoidan is a dietary substance, which represents a class of fucose-enriched sulfated polysaccharides found in the extracellular matrix of brown algae. Numerous experimental evidences indicate that fucoidans display a wide variety of pharmacological activities, including anti-inflammatory, anti-angiogenic, anti-coagulant, and anti-adhesive effects.

### Peer review

The manuscript described that fucoidan is effective to improve intestinal epithelial barrier function. This is an interesting study that could be worth publishing.

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## Antibiotics resistance rate of *Helicobacter pylori* in Bhutan

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### Abstract

**AIM:** To survey the antibiotic resistance pattern of *Helicobacter pylori* (*H. pylori*) strains isolated from Bhutanese population.

**METHODS:** We isolated 111 *H. pylori* strains from the gastric mucosa of *H. pylori*-infected patients in Bhutan in 2010. The Epsilometer test was used to determine the minimum inhibitory concentrations (MICs) of amoxicillin (AMX), clarithromycin (CLR), metronidazole (MNZ), levofloxacin (LVX), ciprofloxacin (CIP), and tetracycline (TET).

**RESULTS:** Nineteen of the isolated *H. pylori* strains were susceptible to all antibiotics tested. The isolated strains showed the highest rate of antibiotic resistance to MNZ (92/111, 82.9%). Among the 92 MNZ-resistant strains, 74 strains (80.4%) showed high-level resistance (MIC  $\geq$  256  $\mu$ g/mL). Three strains were resistance to LVX (2.7%). These strains were also resistance to CIP. None of the strains showed resistance to CLR, AMX and TET.

**CONCLUSION:** CLR-based triple therapy is a more effective treatment approach over MNZ-based triple therapy for *H. pylori* infection in Bhutan.

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**Key words:** *Helicobacter pylori*; Drug resistance; Bhutan

**Core tip:** In Bhutan, 82.9% of *Helicobacter pylori* isolates showed metronidazole resistance. Of these, 80.4% showed high-level resistance (minimum inhibitory concentration  $\geq$  256  $\mu$ g/mL). Only 2.7% strains showed levofloxacin, ciprofloxacin resistance. Intriguingly, none of them were resistance to clarithromycin, amoxicillin, and tetracycline.

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L, Uchida T, Fujioka T, Mahachai V. Antibiotics resistance rate of *Helicobacter pylori* in Bhutan. *World J Gastroenterol* 2013; 19(33): 5508-5512 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5508.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5508>

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a spiral, Gram-negative bacterium that infects more than half of the world's population and is thought to be involved in the pathogenesis of chronic gastritis, peptic ulcer diseases, gastric cancer (GC), and mucosa-associated lymphoid tissue (MALT) lymphoma<sup>[1,2]</sup>. Eradication of *H. pylori* infection not only improves the healing of peptic ulcers, but it also prevents its recurrence and reduces the risk of developing GC<sup>[3-7]</sup>. Furthermore, other *H. pylori*-associated disorders such as MALT lymphoma, atrophic gastritis, and intestinal metaplasia have been shown to regress after treatment with antibiotics<sup>[8-10]</sup>.

Triple therapy regimens that include 1 proton pump inhibitor (PPI) and 2 antimicrobial agents such as amoxicillin (AMX), clarithromycin (CLR), metronidazole (MNZ), levofloxacin (LVX), ciprofloxacin (CIP), and tetracycline (TET) have been widely used to eradicate this bacterium<sup>[7,11,12]</sup>. Although the success of the treatment depends on several factors such as patient compliance and whether the patient is a smoker, antibiotic resistance is the most common factor causing treatment failure<sup>[13-15]</sup>. Prevalence of antibiotic resistance is now increasing worldwide and varies by the geographic area; it is generally higher in developing countries than in developed regions<sup>[16-18]</sup>. In addition, the antibiotic resistance rate often parallels the antibiotic consumption rate in the population<sup>[16,19-21]</sup>.

Bhutan is a small landlocked country in South Asia, located at the eastern end of the Himalayas, and shares its borders in the south, east, and west with the Republic of India and to the north with the People's Republic of China. In Bhutan, the incidence of GC is reported to be quite high (24.2 deaths/100000 population) compared to the neighboring areas<sup>[22]</sup>. Effective therapies to eradicate *H. pylori* can contribute to the decrease of GC incidence in Bhutan. However, information about the prevalence of drug-resistant *H. pylori* strains in Bhutan, which is essential for designing effective eradication therapies, is lacking.

In this study, we aimed to determine the antibiotic susceptibility of *H. pylori* strains isolated from Bhutanese population toward AMX, CLR, MNZ, LVX, CIP, and TET.

## MATERIALS AND METHODS

### Subjects and sample collection

*H. pylori* strains were obtained from the gastric mucosa of *H. pylori*-infected Bhutanese volunteers who under-

went endoscopy at 3 cities within the country (Thimpu, Punaka, and Wangdue) from December 6 to 9, 2010. Biopsy samples from the antrum were endoscopically obtained from each patient and used for culturing *H. pylori* by using standard methods. Cases of peptic ulcers and GC were identified by endoscopy, and GC was further confirmed by histopathology. Gastritis was defined as *H. pylori* infection-mediated gastritis in the absence of peptic ulcer or gastric malignancy. Written informed consent was obtained from all participants, and the protocol was approved by the Ethics Committee of Jigme Dorji Wangchuk National Referral Hospital, Bhutan.

### Drug sensitivity testing

Epsilon meter test (*E* test) was used to determine the minimum inhibitory concentrations (MICs) of AMX, CLR, MNZ, LVX, CIP, and TET. Mueller Hinton II Agar supplemented with 10% horse blood was used as the culture medium and the culture suspension was used to inoculate the agar plates. The *E* test strip of the corresponding antibiotic was placed on the plate and incubated for 3-5 d at 37 °C, under microaerophilic conditions. The MIC was defined by the point of intersection of the inhibition ellipse with the *E* test strip. Strains were considered "resistance" when the MIC values were  $\geq 1$   $\mu\text{g/mL}$  for AMX,  $\geq 1$   $\mu\text{g/mL}$  for CLR,  $\geq 1$   $\mu\text{g/mL}$  for LVX,  $\geq 8$   $\mu\text{g/mL}$  for MNZ, and  $\geq 4$   $\mu\text{g/mL}$  for TET<sup>[23]</sup>. In accordance with previous studies, strains were considered "resistance" to CIP when the MIC values were  $\geq 1$   $\mu\text{g/mL}$ <sup>[24,25]</sup>.

### Statistical analysis

All statistical analyses were performed by SPSS version 19 (SPSS Inc., Chicago, IL, United States). The univariate association between each group was quantified using the unpaired *t* test, Mann-Whitney *U* test, Fisher's exact test, and  $\chi^2$  test. A two-tailed *P* value of  $< 0.05$  was considered statistically significant.

## RESULTS

We isolated 111 strains of *H. pylori* from *H. pylori*-positive Bhutanese patients; the identity of these strains was microbiologically confirmed. The patient group included 51 men and 60 women, with an average age of  $36.8 \pm 13.9$  years. Seventy strains were isolated from patients with gastritis, 11 from patients with peptic ulcer, and 1 from a patient with GC. Nineteen strains were susceptible to all the antibiotics tested. The greatest proportion of isolated strains was resistance to MNZ (92/111, 82.9%, Table 1). The resistance rate was 84.2% (48/57) in the strains isolated from patients younger than 34 years of age, 82.4% (28/34) in the strains isolated from patients aged 35-49 years, 71.4% (10/14) in the strains isolated from patients aged 50-64 years, and 100% (6/6) in the strains isolated from patients above 65 years of age. There was no relationship between the age of the patient and the rate of resistance of the isolated strain to MNZ ( $P = 0.45$ ). Gender was not associated with MNZ resistance as well

**Table 1** Antibiotic susceptibility of 111 *Helicobacter pylori* strains isolated in Bhutan *n* (%)

Total	<i>n</i> = 111
Amoxicillin	0 (0.0)
Clarithromycin	0 (0.0)
Metronidazole	92 (82.9)
Levofloxacin	3 (2.7)
Ciprofloxacin	3 (2.7)
Tetracycline	0 (0.0)

( $P = 0.71$ ). All 11 strains of *H. pylori* from patients with peptic ulcer showed resistance to MNZ; however, it did not statistically differ from the resistance rates of strains isolated from gastritis patients ( $P = 0.11$ ). The distribution of MIC values for MNZ: among 92 MNZ-resistant strains, 74 strains (80.4%) showed high-level resistance (MIC  $\geq 256$   $\mu\text{g/mL}$ ). Three strains (2.7%), from patients with gastritis, were resistant to LVX. These strains were also resistance to CIP. The MIC values for LVX and CIP were 32 and 32  $\mu\text{g/mL}$ , respectively. These 3 strains were also resistance to MNZ. None of the strains showed resistance to CLR, AMX, and TET. Resistance to multiple antibiotics was observed in 3 strains (2.7%), where the bacteria were resistant to MNZ, LVX, and CIP. The strain isolated from the GC patient was susceptible to all the antibiotics tested.

## DISCUSSION

This is the first study exploring the antibiotic resistance pattern of *H. pylori* strains isolated from Bhutanese population. In Bhutan, no domestic guidelines are available for the treatment of *H. pylori* because of insufficient domestic data. At present, the European, Asia-Pacific, and American guidelines on the treatment of *H. pylori* infection recommend a combination of 1 PPI and 2 antibiotics, AMX plus CLR or MNZ, as the first-line therapy<sup>[7,11,12]</sup>. Although lack of patient compliance, inadequate length of therapy, or high bacterial burden are conditions that may contribute to loss of efficacy, antimicrobial resistance is regarded as the leading factor responsible for the failure of eradication of infection. This issue is of particular relevance with regard to CLR, where there can be up to 70% loss of antibiotic effectiveness, depending on macrolide susceptibility *in vitro*<sup>[16]</sup>. Meta-analysis showed that triple therapy consisting of PPI, AMX, and CLR in CLR-resistant infections decreased the treatment efficacy by 66%<sup>[26]</sup>. In fact, the Maastricht III guidelines on *H. pylori* infection management recommend that CLR should not be used when resistance to the antibiotic exceeds 15%-20%<sup>[7]</sup>. However, surprisingly, none of the strains isolated in Bhutan showed resistance to CLR, suggesting that CLR-based triple therapy can still be used to eradicate *H. pylori* in Bhutan. However, resistance to CLR is increasing worldwide with the increase in the use of CLR<sup>[27-29]</sup>, and it is imperative to examine the CLR resistance rate in the *H. pylori* strains in Bhutan.

Resistance to MNZ is extremely high in Bhutan. Recently, the rate of resistance to MNZ has been reported to increase; this can be considered a major factor leading to reduced efficacy of the standard triple therapy in most countries<sup>[11]</sup>. Most MNZ-resistant strains in this study showed a high MIC value ( $\geq 256$   $\mu\text{g/mL}$ ). Regimens including MNZ are not a preferred choice in populations with an MNZ resistance rate of  $> 40\%$ <sup>[19,30]</sup>. Therefore, if CLR resistance increases in future, MNZ cannot be used as a substitute for CLR in the first-line regimen in Bhutan.

MNZ is frequently used to treat not only *H. pylori* infections, but also other infections such as intestinal parasite infections and periodontal and gynecological diseases, which are common in developing countries<sup>[16,31]</sup>. National Statistics Bureau of Bhutan also showed that infectious diarrhea is one of the major causes of mortality in the country, which suggests that MNZ can be often used for its treatment (<http://www.nsb.gov.bt/>).

Recently, LVX has been prescribed as a rescue drug to eradicate infection in case of failure of the first-line therapy<sup>[32,33]</sup>. However, the prevalence of LVX resistance seems to be increasing worldwide and this may reduce the efficacy of treatment with LVX-based regimens<sup>[34-39]</sup>. Therefore, according to the European, Asia-Pacific, and American guidelines, LVX should be used in salvage therapy based on antibiotic susceptibility testing<sup>[7,11,12]</sup>. In Bhutan, LVX is rarely used for the treatment of other infectious diseases and LVX resistance was found only in 3 strains. These strains were also resistant to CIP, suggesting cross-resistance among the fluoroquinolone drugs. TET resistance was not noted in any of the strains tested, consistent with the findings of previous studies from other countries<sup>[25,40,41]</sup>. TET is not often used for the treatment of infectious diseases in Bhutan. Therefore, TET-based or quadruple therapy including TET can be a useful alternative to the first-line regimen, as recommended in the European and Asia-Pacific guidelines<sup>[7,11]</sup>. Likewise, all strains in this study were susceptible to AMX, which is consistent with previous findings<sup>[40,42,43]</sup>.

However, we should be cautious about implementing the eradication therapy in Bhutan. Despite the success of the *H. pylori* eradication therapy, the infection does frequently recur in patients in developing countries where there is a high prevalence of *H. pylori* infection<sup>[44]</sup>. Such repeat infection is either a recurrence of the original infection or reinfection with a new strain. Environmental factors, including poor living conditions, are related to high rates of *H. pylori* infection<sup>[45,46]</sup>. In rural areas of the country, river or pond water can be used as the source of drinking water (information from National Statistics Bureau, <http://www.nsb.gov.bt/>); furthermore, unsanitary pit latrines are widely used in this country. It is necessary to improve sanitary conditions to decrease the prevalence of *H. pylori*.

In conclusion, CLR-based triple therapy can still be used to eradicate *H. pylori* in Bhutan. However, because of high resistance rates, MNZ-based triple therapy is not useful as the first-line therapy. It is necessary to have

current and reliable information on the prevalence of antibiotic resistance to *H. pylori*, in particular, in Bhutan. Careful consideration is required for formulating national therapeutic guidelines for the first-line and second-line therapies for *H. pylori* infection, considering factors such as disease prevalence, access to health care centers, diagnostic facilities, and the burden of health care costs borne by the government.

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## COMMENTS

### Background

Eradication of *Helicobacter pylori* (*H. pylori*) reduces the risk of developing gastric cancer (GC). Antibiotic resistance is the most common factor causing the failure of the treatment. Prevalence of antibiotic resistance is now increasing worldwide and varies in geographic area. The incidence of GC in Bhutan is reported to be quite high comparing with neighbor area. Therefore, effective eradication therapy can contribute to the decrease of incidence of GC in Bhutan. However, the prevalence of drug resistant *H. pylori* in Bhutan has not been elucidated.

### Research frontiers

Triple therapy regimens including one proton pump inhibitor and two antimicrobial agents such as amoxicillin (AMX), clarithromycin (CLR), metronidazole (MNZ), levofloxacin (LVX), ciprofloxacin (CIP), and tetracycline have been widely used to eradicate *H. pylori*. Prevalence of antibiotic resistance is now increasing worldwide and varies in geographic area. Although the success of the treatment depends on several factors such as smoking and patient compliance, antibiotic resistance is the most common factor causing the failure of the treatment. Therefore, it is necessary to examine the recent drug resistance rates to select the proper eradication regimens.

### Innovations and breakthroughs

Although the incidence of GC in Bhutan is quite high comparing with neighbor area, the prevalence of drug resistant *H. pylori* in Bhutan has not been elucidated. The author's findings can contribute to the decrease of incidence of GC in Bhutan.

### Applications

CLR-based triple therapy can be used to eradicate *H. pylori* whereas MNZ-based triple therapy is not suitable for *H. pylori* eradication in Bhutan.

### Peer review

The manuscript reports on the pattern of *H. pylori* resistance to antibiotics in Bhutan. The strains of *H. pylori*, cultured from antral mucosal biopsies 111 patients, were assessed for susceptibility to GC, clarithromycin, metronidazole, levofloxacin, ciprofloxacin, and tetracycline, using Epsilometer test. The results revealed the highest resistance rate (83%) to MNZ followed by LVX and CIP, both about 2.7%. This manuscript of limited importance, and of interest to those studying the health status of Bhutanese.

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## Self-expandable metallic stent placement plus laparoscopy for acute malignant colorectal obstruction

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January 2010 and December 2011 to explore whether SEMS placement influenced the laparoscopic procedure or reduced long-term survival by influencing CRC oncological characteristics.

**RESULTS:** The characteristics of patients among these groups were comparable. The rate of conversion to open surgery was 12.5% in the stent-laparoscopy group. Bowel function recovery and postoperative hospital stay were significantly shorter ( $3.3 \pm 0.9$  d vs  $4.2 \pm 1.5$  d and  $6.7 \pm 1.1$  d vs  $9.5 \pm 6.7$  d,  $P = 0.016$  and  $P = 0.005$ ), and surgical time was significantly longer ( $152.1 \pm 44.4$  min vs  $127.4 \pm 38.4$  min,  $P = 0.045$ ) in the stent-laparoscopy group than in the stent-open group. Surgery-related complications and the rate of admission to the intensive care unit were lower in the stent-laparoscopy group. There were no significant differences in the interval between stenting and surgery, intraoperative blood loss, OS, and DFS between the two stent groups. Compared with those in the stent-laparoscopy group, all surgery-related parameters, complications, OS, and DFS in the control group were comparable.

**CONCLUSION:** The stent-laparoscopy approach is a feasible, rapid, and minimally invasive option for patients with ACO caused by left-sided CRC and can achieve a favorable long-term prognosis.

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**Key words:** Self-expandable metallic stent; Colorectal cancer; Endoscopy; Laparoscopy; Efficiency; Safety

**Core tip:** Our study compared long-term survival between left-sided colorectal cancer (CRC) patients with acute colorectal obstruction (ACO) who had undergone self-expandable metallic stent (SEMS) placement followed by one-stage laparoscopic (stent-laparoscopy

### Abstract

**AIM:** To investigate the clinical advantages of the stent-laparoscopy approach to treat colorectal cancer (CRC) patients with acute colorectal obstruction (ACO).

**METHODS:** From April 2008 to April 2012, surgery-related parameters, complications, overall survival (OS), and disease-free survival (DFS) of 74 consecutive patients with left-sided CRC presented with ACO who underwent self-expandable metallic stent (SEMS) placement followed by one-stage open ( $n = 58$ ) or laparoscopic resection ( $n = 16$ ) were evaluated retrospectively. The stent-laparoscopy group was also compared with a control group of 96 CRC patients who underwent regular laparoscopy without ACO between

group) and open resection (stent-open group). Long-term survival in left-sided CRC patients without ACO who had undergone laparoscopic resection (control group) was compared with the stent-laparoscopy group. A stent-laparoscopy approach did not reduce long-term survival by influencing CRC oncological characteristics. Surgery-related parameters and postoperative complications in the stent-laparoscopy group were also compared with those of the other two groups; the results indicated that SEMS placement did not influence subsequent laparoscopic procedures.

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## INTRODUCTION

Acute colorectal obstruction (ACO) is one of the common initial symptoms in patients with left-sided colorectal cancer (CRC). Emergent surgery is a conventional treatment, but it is usually associated with high morbidity, mortality, and stoma rate<sup>[1-3]</sup>. Since 1991, self-expanding metallic stent (SEMS) placement has been applied to relieve ACO caused by left-sided CRC and is effective in restoring colorectal transit, allowing sufficient preoperative preparation and tumor stage evaluation<sup>[4-6]</sup>. Compared with emergent surgery, preoperative stenting and elective surgery are safer and increase the probability of one-stage resection<sup>[7,8]</sup>. Open and laparoscopic colectomies are two recent approaches used as a subsequent elective surgery following successful SEMS placement. Laparoscopic colectomy has a lower postoperative complication rate and a shorter hospital stay<sup>[9]</sup>. The application of SEMS placement can increase the probability of performing laparoscopic colectomy and offers the advantages of two minimally invasive procedures<sup>[10]</sup>. The stent-laparoscopy approach was first introduced by Morino *et al*<sup>[11]</sup> in 2002, and its use has been reported in previous studies<sup>[12-18]</sup>. In Morino's study, preoperative SEMS placement was believed to make the laparoscopic procedure more difficult and the colonic segment more bulky and more technically difficult to remove through laparoscopy<sup>[10]</sup>, but this has not been confirmed. Similarly, the long-term survival of patients undergoing stent-laparoscopy is currently unknown. Therefore, the present study was designed to compare surgery-related parameters, including surgical time and intraoperative blood loss, postoperative complications, long-term overall survival (OS) and disease-free survival (DFS), of the stent-laparoscopy approach with the stent-open surgery approach in left-sided CRC patients with ACO and with regular laparoscopy in left-sided CRC patients without ACO to determine the clinical advantages and long-term prognoses of the stent-

laparoscopy approach and the influence of preoperative SEMS placement on the laparoscopic procedure.

## MATERIALS AND METHODS

### *Patients and follow-up*

From April 2008 to April 2012, 74 consecutive patients (47 males and 27 females, aged 34-84 years, median 60 years) with left-sided CRC and ACO, who had undergone SEMS placement followed by one-stage resection at Zhongshan Hospital, were reviewed retrospectively. The obstruction was diagnosed clinically and radiologically. Patient symptoms were abdominal pain and fullness, vomiting and constipation. Physical examination showed a distended and tympanic abdomen. Abdominal X-ray revealed a distended large bowel and an air-fluid level. All patients underwent endoscopic SEMS placement to release the obstruction. According to the particular subsequent resection approach selected by the attending surgeon, patients were allocated into the stent-laparoscopy group and the stent-open group. Additionally, from January 2010 to December 2011, 96 left-sided CRC patients without ACO who had undergone one-stage laparoscopic resection were enrolled consecutively as the control group. All patients were enrolled after informed consent. The Research Ethics Committee of Zhongshan Hospital approved the study.

After surgery, the follow-up procedures in the stent-surgery groups, including chest X-ray, abdominal ultrasound, computed tomography scan and blood tests, especially levels of cancer embryo antigen, were performed every 3 to 4 mo within 2 years, and continued every 4 to 6 mo for 3 to 4 years thereafter. Colonoscopy was performed every 6 mo in the first year and every year for 2 to 4 years. Tumor, node, metastasis (TNM) staging was performed according to the American Joint Committee on Cancer, 6<sup>th</sup> edition. OS was defined as the interval between SEMS placement and death or the last follow-up visit. DFS was defined as the interval between SEMS placement and recurrence or postoperative remote organ metastasis. If recurrence was not diagnosed, patients were censored on the date of death or last follow-up.

### *Endoscopic stenting procedure and laparoscopic resection*

Briefly, all SEMS placement procedures were performed by one of five experienced endoscopists using a colonoscope (CF-260I; Olympus, Tokyo, Japan) with fluoroscopic guidance. Water-soluble contrast material was injected through the catheter to visualize the stricture. The size of the SEMS was selected according to the length and caliber of the stricture (diameter, 26 mm; length, range 50-100 mm). The length of the SEMS was at least an additional 2 cm on each side of the stricture. A SEMS from MicroTech (MicroTech Co., Nanjing, China) or Boston Scientific (Boston Scientific, Natick, MA, United States) was used according to the endoscopist's preference. After deployment, the proper position and

**Table 1** Characteristics of the patients in two groups

Characteristics	Stent-laparoscopy	Stent-open	Control
Conversion to open surgery <i>n</i> (%)	2 (12.5)	-	8 (8.3)
Patients	14	58	88
Age (yr)	57.7 ± 9.6	60.2 ± 12.8	59.6 ± 10.1
Gender (male/female)	10/4	36/22	53/35
Site of obstruction			
Descending colon	4	15	13
Sigmoid colon	7	26	51
Rectum	3	17	24
TNM stage			
I	0	1	5
II	6	24	37
III	5	21	27
IV	3	12	19

TNM: Tumor, node, metastasis.

expansion of the SEMS was assessed using fluoroscopic visualization.

After complete remission of ACO, bowel preparation was performed with polyethylene glycol 24 h before surgery. Patients were placed in the Trendelenburg position, and slightly tilted to the right and downward. The target colorectum and its mesentery were mobilized laparoscopically, and the colon distal to the tumor was divided using endo linear staplers. A vertical periumbilical incision was made to remove the specimen and introduce the anvil of the circular stapler. An anastomosis was made in an end-to-end manner using the circular stapler or in a side-to-side manner using the double staples method to discriminate the size of the colorectal lumen.

### Statistical analysis

Values are expressed as the mean ± SD. An unpaired *t* test was used to compare quantitative variables. A Pearson's  $\chi^2$  test or Fisher's exact test was applied to compare qualitative variables. The patients' survival curve was plotted using the Kaplan-Meier method, and the log-rank test was used to determine the significant differences between groups. Analysis was performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, United States). A *P* value less than 0.05 was considered statistically significant. All images were edited using Photoshop CS5 extended (Adobe, San Jose, CA, United States).

## RESULTS

### Patient characteristics

Sixteen patients were in the stent-laparoscopy group and 58 patients were in the stent-open group. In the stent-laparoscopy group, two patients (12.5%) converted to open surgery for abdominal carcinomatosis and serious local intestinal adhesions; both conditions were unrelated to the stenting. In the control group, the rate of conversion to open surgery was 8.3% (8/96); three for serious local intestinal adhesions, four for extensive tissue invasion of the tumor and one for an inappropriate tumor site, which

**Table 2** Characteristics and outcomes

	Stent-laparoscopy	Stent-open	Control
Interval between stenting and surgery (d)	13.9 ± 13.2	10.6 ± 13.3	-
Operation time (min)	152.1 ± 44.4	127.4 ± 38.4 <sup>a</sup>	152.3 ± 40.8
Intraoperative blood loss (mL)	54.3 ± 63.0	77.4 ± 132.7	77.1 ± 41.4
Bowel function recovery (d)	3.3 ± 0.9	4.2 ± 1.5 <sup>a</sup>	3.1 ± 0.7
Postoperative hospital stay (d)	6.7 ± 1.1	9.5 ± 6.7 <sup>a</sup>	6.3 ± 3.5
Admitted to ICU <i>n</i> (%)	0 (0.0)	12 (20.7)	8 (9.1)
Postoperative complications			
Incision rupture	0	2	0
Incision infection	0	2	1
Anastomotic leakage	0	2	0
Adhesive intestinal obstruction	0	1	0
Postoperative stroke	0	0	1

<sup>a</sup>*P* < 0.05 vs control group. ICU: Intensive care unit.

was not significantly different from the stent-laparoscopy group. These patients were excluded from the analyzed data. The patient characteristics were comparable among the three groups (Table 1).

### Comparison of clinical outcomes

The mean interval between stenting and surgery in the stent-open and stent-laparoscopy groups were 10.6 and 13.9 d, respectively (*P* = 0.397), and 8.8 and 10.2 d (*P* = 0.162), respectively, after the patients who received preoperative chemotherapy were excluded. No intraoperative morbidity was observed in either group. The mean surgical time in the stent-laparoscopy group was significantly longer than in the stent-open group (152.1 min vs 127.4 min, *P* = 0.045). However, intraoperative blood loss was not significantly different (*P* = 0.530). After surgery, mean bowel function recovery and postoperative hospital stay in the stent-laparoscopy group were significantly shorter than those in the stent-open group (3.3 d vs 4.2 d and 6.7 d vs 9.5 d, *P* = 0.016 and *P* = 0.005, respectively). In the stent-open group, 20.7% (12/58) of patients were admitted to the intensive care unit (ICU) after surgery, whereas none were admitted to the ICU in the laparoscopy group. No postoperative complications were observed in the stent-laparoscopy group, whereas seven patients (12.1%) with postoperative complications were observed in the stent-open group (Table 2).

Compared with the control group, surgery-related parameters, including surgical time, intraoperative blood loss, bowel function recovery, and postoperative hospital stay, were comparable in the stent-laparoscopy group. In the control group, eight patients (9.1%) were admitted to the ICU after surgery and postoperative complications occurred in two patients (2.3%) (Table 2).

### Comparison of long-term survival

The long-term survival of patients in the three groups was investigated. The follow-up period of patients in the stent-laparoscopy group was 28.2 ± 13.0 mo, which was not significantly different from that of the stent-

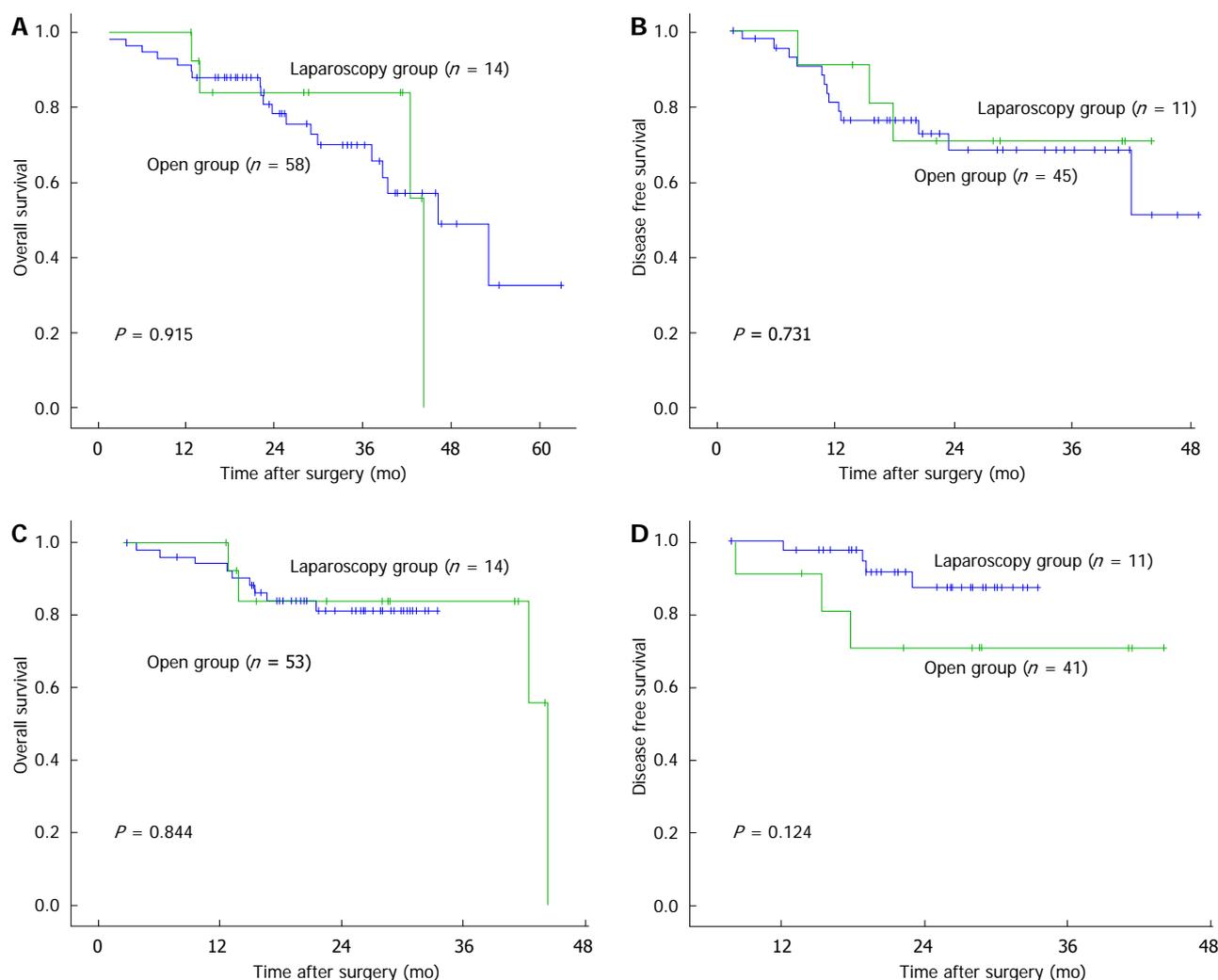


Figure 1 Kaplan-Meier analysis. A and B: In the stent-laparoscopy (green line) and stent-open (blue line) groups, there were no significant differences in overall survival and disease-free survival ( $P = 0.915$  and  $P = 0.731$ ); C and D: In the stent-laparoscopy (green line) and control (blue line) groups, there were also no significant differences in overall survival and disease-free survival ( $P = 0.844$  and  $P = 0.124$ ).

open group ( $28.9 \pm 13.8$  mo,  $P = 0.865$ ) and the control group ( $22.2 \pm 7.9$  mo,  $P = 0.118$ ), respectively. Patients with TNM stage IV were excluded from the DFS analysis. The 1-, 2-, 3-, and 4-year OS and DFS of patients in the stent-laparoscopy group were 100%, 83%, 83%, and 36%, and 91%, 71%, 71%, and 71%, respectively, which were not significantly different from those of the stent-open group (91%, 79%, 70%, and 50%; and 82%, 70%, 70%, and 57%, respectively,  $P = 0.915$  and  $P = 0.731$ ; Figure 1A and B). The 1-, 2-, and 3-year OS and DFS of patients in the control group were 94%, 80%, and 80%, and 100%, 88%, and 88%, respectively ( $P = 0.844$  and  $P = 0.124$ ; Figure 1C and D), which were also not significantly different from those of the stent-laparoscopy group. At the end of this study, 10 patients in the stent-laparoscopy group, 39 patients in the stent-open group, and 44 patients in control group remained alive. The details of recurrence, metastasis, and treatment are shown in Table 3 (Figure 2 shows a surgical specimen containing an SEMS).

## DISCUSSION

Malignant ACO was considered a relative contraindication of laparoscopy because of an unprepared fragile bowel and insufficient working space caused by the distended bowel, until SEMS placement was extended from a palliative treatment to a “bridge to surgery” treatment<sup>[19]</sup>. Meanwhile, the surgical approach to malignant ACO has changed extensively over time. The traditional three-stage operation was replaced gradually by a one-stage resection with primary anastomosis<sup>[11]</sup>. Preoperative SEMS placement also dramatically increases the probability of subsequent one-stage resection, using either an open or laparoscopic approach<sup>[7,20]</sup>. Morino *et al.*<sup>[11]</sup> first reported four left-sided CRC patients with ACO who were treated by a stent-laparoscopy approach. Although positive conclusions could be drawn, the lack of both an appropriate control group and long-term outcomes, as well as a limited number of patients made further study necessary. Likewise, other studies reported conflicting re-

**Table 3** Recurrence, metastasis and treatment

	Stent-laparoscopy	Stent-open <sup>1</sup>	Control <sup>2</sup>
Recurrence	1	5	-
Metastasis			
Liver	-	3	1
Lung	1	2	-
Uterus	1	-	-
Brain	-	1	-
Pelvic cavity	-	1	3
Multiple organs	-	1	-
Treatment			
Surgery	1	3	1
Chemotherapy	1	8	1
Radiotherapy	1	1	-

<sup>1</sup>One patient's treatment was unknown; <sup>2</sup>Two patients' treatments were unknown.

sults. Thus, the present larger, long-term follow-up study aimed to report our experience and discuss the issues in previous studies by comparing the stent-laparoscopy approach with the stent-open approach and regular laparoscopy.

As the first step of the stent-laparoscopy approach, preoperative SEMS placement generally has a high success rate<sup>[8]</sup>. The technical and clinical success rates of SEMS placement are more than 96.7% and more than 90%, respectively. Moreover, no SEMS placement-associated morbidity or mortality was observed<sup>[11-17]</sup>. The technical and clinical success rates of patients in our center since 2005 were also similar to the data in these previous studies. A high success rate and low risk of preoperative SEMS placement guarantee the feasibility of the stent-laparoscopy approach.

For the laparoscopy procedure, several controversial issues have been reported in previous studies. Balagué *et al*<sup>[12]</sup> first suggested that the rigidity of the colonic segment containing the stent and the tumor made dissection more difficult than usual, prolonging the surgical time. In the same year, Law *et al*<sup>[13]</sup> reported that laparoscopic mobilization was not particularly difficult and the amount of blood loss was low. Following these studies, the results of study of Chung *et al*<sup>[17]</sup> partly supported Law's conclusions; the data from eight stent-open group patients were similar to those of the 17 stent-laparoscopy patients in terms of surgical time, estimated blood loss, and other surgery-related and postoperative parameters. In our study, intraoperative blood loss was not significantly different between the stent-laparoscopy and stent-open approaches, or regular laparoscopy, supporting Chung's conclusions and indicating the favorable safety of the stent-laparoscopy approach. However, the surgical times were not completely consistent with those reported in the above-mentioned studies. We found that compared with the stent-open approach, the stent-laparoscopy approach significantly prolonged the surgical time. When we compared the stent-laparoscopy approach with regular laparoscopy, no significant differences in the surgical times between these two groups were observed. Moreover, the rate of conver-



**Figure 2** Surgical specimen containing a preoperatively placed self-expandable metallic stent.

sion to open surgery in the stent-laparoscopy approach was 12.5%, which was similar to 8.3% in regular laparoscopy, and the two causes of conversion were related to the tumor or abdominal conditions (tumor invasion and intestinal adhesions), but unrelated to preoperative SEMS placement. Thus, we confirmed that the major influences on subsequent surgical procedures after stenting were the difficulties from the laparoscopy itself, and tumor or abdominal conditions, but not preoperative SEMS placement. Additionally, skilled surgeons performed all of the surgical procedures in our study, so a technical bias could be excluded.

Regarding postoperative recovery, bowel function recovery and postoperative hospital stays for the stent-laparoscopy group were significantly shorter than those for the stent-open group, and were similar to those of the regular laparoscopy group. Furthermore, no postoperative complications were observed for the stent-laparoscopy group, which was similar to that of the regular laparoscopy group, but fewer than the 12.1% in the stent-open group. Our results also indicated that using the stent-laparoscopy approach was associated with faster recovery and lower postoperative morbidity, which was similar to the results of previous studies<sup>[16,17,21-23]</sup>.

Long-term survival in these three groups was compared to estimate the curative effect of the stent-laparoscopy approach. Previously, Stipa *et al*<sup>[6]</sup> reported that their minimum follow-up period was 15 mo, and 17/22 (77%) surgically treated patients (six patients in the stent-open group and 16 in the stent-laparoscopy group) were alive at the end of their study. In Dulucq's study, neither recurrences nor port-site metastases were observed during a follow-up period of  $11 \pm 7$  mo<sup>[14]</sup>. Similarly, Olmi *et al*<sup>[15]</sup> reported that after a median 36-mo follow-up period, all 19 patients in the stent-laparoscopy group and four patients in the stent-open group were alive. The superiority of laparoscopic colectomy for treating malignancy over open surgery in terms of recurrence and cancer-related survival was demonstrated in a previous randomized trial<sup>[24]</sup>. In several recent, large-scale randomized control trials (RCTs), no significant differences in 3- or 5-year OS and DFS between laparoscopy and open surgery were ob-

served<sup>[21]</sup>. In the present study, 4-year OS and DFS were compared between laparoscopy and open surgery after SEMS placement, and no significant differences were observed, in accordance with the results of these RCTs. Thus, we suggest that different surgical approaches after stenting do not influence long-term clinical outcomes. On the other hand, no study had explored whether a preoperative SEMS influenced the curative effect of subsequent laparoscopy for exacerbating CRC oncological characteristics, such as promoting recurrence or metastasis. Therefore, we compared long-term OS and DFS between the stent-laparoscopy approach and regular laparoscopy, and no significant differences were observed. These results indicate that preoperative SEMS placement is completely safe for subsequent laparoscopy.

In conclusion, compared with the stent-open approach, the stent-laparoscopy approach was associated with a more difficult surgical procedure, but a faster postoperative recovery and lower morbidity. These two approaches show similar long-term survival, recurrence rates and metastasis rates. Furthermore, after comparison with regular laparoscopy, preoperative SEMS placement does not influence subsequent laparoscopic procedures and long-term survival could be assessed. Therefore, SEMS placement followed by one-stage laparoscopic surgery is a feasible and rapid recovery treatment option for patients with ACO caused by left-sided CRC, and provides a favorable long-term prognosis. Of course, this study is limited by the patients' conditions and the study method employed; thus, heterogeneity among the groups in our study cannot be excluded. A larger number of patients, a longer follow-up period and more homogeneous study groups should be included in a future study.

## COMMENTS

### Background

Acute colorectal obstruction (ACO) is a common initial symptom in patients with left-sided colorectal cancer (CRC). Placement of a self-expanding metallic stent (SEMS), followed by elective surgery, will gradually replace conventional treatment (emergent surgery), and become the predominant treatment. Meanwhile, the application of a SEMS enhances the need for laparoscopic colectomy, avoiding colostomy, and offers the advantages of two minimally invasive procedures.

### Research frontiers

SEMS placement followed by laparoscopy is an emerging and accepted treatment for left-sided CRC patients with ACO. However, preoperative SEMS placement is believed to make the laparoscopic procedure more difficult, because the SEMS make the colonic segment more bulky and more technically difficult to remove *via* laparoscopy. In addition, long-term clinical outcomes of the stent-laparoscopy approach are unknown.

### Innovations and breakthroughs

Authors compared surgery-related parameters, postoperative complications, and long-term survival using the stent-laparoscopy approach with those using the stent-open approach and regular laparoscopy in left-sided CRC patients without ACO. They first reported that preoperative SEMS placement did not influence any subsequent laparoscopic procedure. Furthermore, using a stent-laparoscopy approach could achieve a similar curative effect compared with the other two approaches and did not reduce long-term survival of patients by influencing CRC oncological characteristics.

### Applications

The results indicate that SEMS placement followed by one-stage laparoscopic surgery is a feasible treatment option for left-sided CRC patients with ACO,

and shows rapid recovery. Moreover, the treatment does not reduce long-term survival by influencing CRC oncological characteristics, which confirms the curative effect of the stent-laparoscopy approach and may allow it to be applied more widely.

### Terminology

SEMS placement: a SEMS is placed in the stricture of the ACO to drain the excrement.

### Peer review

This study details the clinical outcomes and long-term survival of patients undergoing preoperative SEMS placement and laparoscopy. The results show that the stent-laparoscopy approach is a feasible treatment for left-sided CRC patients with ACO, with rapid recovery and good long-term prognosis.

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## Diffusion-weighted magnetic resonance imaging for predicting the response of rectal cancer to neoadjuvant concurrent chemoradiation

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### Abstract

**AIM:** To evaluate the clinical value of diffusion-weighted magnetic resonance imaging (DW-MRI) in predicting the response of rectal cancer to neoadjuvant chemoradiation.

**METHODS:** This prospective study was approved by our institutional review board, and informed consent was obtained from each patient. Fifteen patients (median age 56 years) with locally advanced rectal cancer were treated in our hospital from June 2006 to December 2007. All patients were stage III B-C accord-

ing to the results of MRI and endorectal ultrasound examinations. All patients underwent pelvic irradiation with 45 Gy/25 fx per 35 days. The concurrent chemotherapy regimen consisted of capecitabine 625 mg/m<sup>2</sup>, *bid* (Monday-Friday), and oxaliplatin 50 mg/m<sup>2</sup>, weekly. The patients underwent surgery 5-8 wk after the completion of neoadjuvant therapy. T downstaging was defined as the downstaging of the tumor from cT3 to ypT0-2 or from cT4 to ypT0-3. Good regression was defined as TRG 3-4, and poor regression was defined as TRG 0-2. Diffusion-weighted magnetic resonance images were obtained prior to and weekly during the course of neoadjuvant chemoradiation, and the apparent diffusion coefficient (ADC) values were calculated from the acquired tumor images.

**RESULTS:** Comparison with the mean pretreatment tumor ADC revealed an increase in the mean tumor ADC during the course of neoadjuvant chemoradiation, especially at the 2<sup>nd</sup> week ( $P = 0.004$ ). We found a strong negative correlation between the mean pretreatment tumor ADC and tumor regression after neoadjuvant chemoradiation ( $P = 0.021$ ). In the T downstage and tumor regression groups, we found a significant increase in the mean ADC at the 2<sup>nd</sup> week of neoadjuvant therapy ( $P = 0.011$ ; 0.004).

**CONCLUSION:** DW-MRI might be a valuable clinical tool to help predict or assess the response of rectal cancer to neoadjuvant chemoradiation at an early time-point.

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**Key words:** Locally advanced rectal cancer; Neoadjuvant chemoradiation; Diffusion-weighted magnetic resonance imaging; Apparent diffusion coefficient

**Core tip:** This original study prospectively evaluated the

clinical value of diffusion-weighted magnetic resonance imaging (DW-MRI) in predicting the response of rectal cancer to neoadjuvant chemoradiation. We found a strong negative correlation between the mean pretreatment tumor apparent diffusion coefficient (ADC) and tumor regression after neoadjuvant chemoradiation, as well as a significant increase in the mean ADC at the 2<sup>nd</sup> week in the T downstage and tumor regression groups. Therefore, DW-MRI might be a valuable clinical tool to help predict or assess the response of rectal cancer to neoadjuvant chemoradiation at an early timepoint.

Cai G, Xu Y, Zhu J, Gu WL, Zhang S, Ma XJ, Cai SJ, Zhang Z. Diffusion-weighted magnetic resonance imaging for predicting the response of rectal cancer to neoadjuvant concurrent chemoradiation. *World J Gastroenterol* 2013; 19(33): 5520-5527 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5520.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5520>

## INTRODUCTION

Neoadjuvant (chemo) radiation followed by total mesorectal excision has become the standard treatment for locally advanced rectal cancer (LARC)<sup>[1-3]</sup>. However, approximately 20%-30% of patients do not benefit from neoadjuvant treatment due to the radioresistance of the tumor<sup>[4]</sup>, and ineffective neoadjuvant treatment may result in unnecessary toxicity and expense as well as delays in receiving the proper treatment. Meanwhile, 10%-30% of patients with a pathological complete response (pCR) have a favorable long-term outcome<sup>[5]</sup>. Recently, data have even suggested that surgery is unnecessary for clinical complete responders<sup>[6]</sup>. To effectively guide patient-tailored treatments, reliable and early assessment of the treatment response is important.

Diffusion-weighted magnetic resonance imaging (DW-MRI) is a non-invasive functional MRI technique that is sensitive to the mobility of water protons in biological tissues, which is dependent on many factors, such as cell density, vascularity, the viscosity of the extracellular fluid, and cell membrane integrity<sup>[7-9]</sup>. The apparent diffusion coefficient (ADC) calculated from DW-MRI measurements can quantify and express these properties. However, published data on the value of DW-MRI as a predictive tool for anti-cancer treatment responses in patients with rectal cancer are scarce and conflicting. Most studies have found that the pretreatment ADC is negatively correlated with the response to treatment<sup>[10]</sup>. Furthermore, it is possible that necrotic areas with high pretreatment ADCs are less sensitive to radiation and chemotherapy, although several studies do not support this hypothesis, and others have obtained opposite results<sup>[11,12]</sup>. Therefore, we conducted this study to investigate the clinical value of DW-MRI as a predictor of the tumor response in patients receiving neoadjuvant chemoradiation therapy (CRT) for rectal cancer by measuring the tumor ADC.

## MATERIALS AND METHODS

Our institutional review board approved this prospective study, and informed consent was obtained from each patient.

### Patients and treatment

Fifteen patients (median age 56 years, range 32-69 years; 13 men and 2 women) with LARC were invited to participate in our study between June 2006 and December 2007. Each patient had histologically proven rectal adenocarcinoma of stage T3-T4 and was determined to be node-positive by endorectal ultrasound and pelvic MRI. Patients with a history of pelvic irradiation or chemotherapy, any other malignancy, or distant metastases were excluded (Table 1). The clinical and histopathological classification and stage according to the International Union Against Cancer TNM system<sup>[13]</sup> were recorded. Tumor regression grading was evaluated according to the criteria of Dworak *et al.*<sup>[14]</sup> (grade 0, no regression; grade 1, minor regression, dominant tumor mass with obvious fibrosis in 25% or less of the tumor mass; grade 2, moderate regression, 26%-50% of the tumor mass; grade 3, good regression, more than 50% tumor regression; and grade 4, total regression, no viable tumor cells, only fibrotic mass). A pCR was defined as the absence of viable tumor cells in the primary tumor and lymph nodes (ypT0N0). T downstaging was defined as the downstaging of the tumor from cT3 to ypT0-2 or from cT4 to ypT0-3. Good regression was defined as TRG 3-4, and poor regression was defined as TRG 0-2.

All patients received neoadjuvant concurrent CRT. Radiotherapy (RT) was delivered with a linear accelerator using 6- and 15-MV photons and a three-field technique (posterior-anterior and right and left laterals). Every patient underwent a planning computed tomography (CT) scan in the treatment position (prone position) using a belly board. Three-dimensional conformal RT was used for all patients based on the planning CT, with a total dose of 45 Gy at 1.8 Gy per fraction per day, Monday-Friday. Neoadjuvant chemotherapy was delivered concurrently with RT. Starting on day 1 of RT, patients received capecitabine 625 mg/m<sup>2</sup> orally, *bid* (Monday-Friday), and oxaliplatin 50 mg/m<sup>2</sup> weekly for five consecutive weeks. Surgical resection was scheduled for 5-8 wk after the completion of neoadjuvant treatment.

### DW-MRI

Each enrolled patient was examined by DW-MRI at six scheduled times. The initial DW-MRI scan was performed 7 d prior to the start of RT. DW-MRI scans were then taken once weekly during the course of neoadjuvant treatment.

DWI was performed on a 1.5 T magnetic resonance machine (1.5 T Signa Twin Speeder with Excite, GE, United States) using a phased-array body coil. Before DW-MRI, standard T2-weighted fast spin echo sequence and T1-weighted spin echo sequence images were used for clinical staging. DWI echo planar images were ac-

**Table 1 Patients and treatment characteristics**

No.	Age (yr)	Preoperative stage	Surgical treatment	Postoperative stage
1	56	cT3N2M0	LAR	ypT0N1M0
2	57	cT4N2M0	LAR	ypT3N0M0
3	46	cT3N2M0	LAR	ypT3N1M0
4	69	cT4N1M0	APR	ypT2N0M0
5	40	cT3N2M0	APR	ypT3N2M0
6	40	cT3N1M0	APR	ypT0N0M0
7	58	cT4N1M0	APR	ypT2N0M0
8	57	cT3N2M0	APR	ypT0N0M0
9	51	cT4N2M0	Exploratory laparotomy	ypT4N2M0
10	55	cT3N1M0	APR	ypT3N1M0
11	68	cT3N2M0	APR	ypT3N0M0
12	58	cT3N2M0	APR	ypT3N1M0
13	61	cT3N2M0	LAR	ypT1N1M0
14	32	cT4N2M0	APR	ypT3N1M0
15	55	cT3N1M0	APR	ypT3N1M0

LAR: Low anterior resection; APR: Abdominal perineal resection.

quired in the transverse plane using a GRE-EPI sequence (TR/TE 3000/min; field of view 22 cm<sup>2</sup>; matrix size 128 × 128; slice thickness 4 mm; intersection gap 1 mm). DW-MR images and ADC maps were obtained using *b* values of 0 and 1000 s/mm<sup>2</sup> applied in the *x*, *y*, and *z* directions. Patients did not undergo bowel preparation, receive anti-spasmodic medication, or undergo rectal distention before the MR examination. For the image analysis, the data were transferred to a Workstation (AW4.0, GE Medical Systems) and analyzed using the Functool dynamic analysis tool (GE Medical Systems). ADC values were calculated based on the ADC maps. The ADC map of the largest tumor extension in the transverse T2-weighted images was used for the analysis. Regions of interest (ROIs) were drawn manually along the edge of the tumor with a *b* value of 1000 s/mm<sup>2</sup> on the selected ADC maps by an experienced radiologist (Zhang S, with 10 years of experience in clinical MRI), who did not participate in the treatment of the patients or the evaluation of the therapeutic effect.

### Statistical analysis

Statistical analysis was performed using SPSS 12.0 statistical software. Paired comparisons were performed using the Wilcoxon test. Spearman's correlation was used to assess the significance of differences between groups. A *P* value < 0.05 was considered statistically significant.

## RESULTS

### Treatment characteristics

After neoadjuvant treatment, pCR was observed in 2 patients. Downstaging of the tumor was observed in eight patients. The tumor regression grades after neoadjuvant treatment were grade 0-2 in 6 patients and grade 3-4 in 9 patients.

### DW-MRI data

Diffusion data from 15 patients were obtained prior to

and at constant intervals once weekly during the course of neoadjuvant treatment. The observed ADC values are shown in Table 2. A total of 88 ADC values were obtained in our study, and 2 ADC values were excluded due to measurement errors. Sample T2-weighted and diffusion-weighted images prior to treatment are shown in Figure 1. Sample ADC maps from the images taken weekly during the course of neoadjuvant treatment are shown in Figure 2.

The mean tumor ADC value slightly increased from  $0.749 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.641 \times 10^{-3}$ - $0.858 \times 10^{-3}$  mm<sup>2</sup>/s) prior to treatment to  $0.772 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.627 \times 10^{-3}$ - $0.918 \times 10^{-3}$  mm<sup>2</sup>/s) after the 1<sup>st</sup> week of treatment. There was also a significant increase at the 2<sup>nd</sup> week to  $0.884 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.775 \times 10^{-3}$ - $0.994 \times 10^{-3}$  mm<sup>2</sup>/s). Subsequently, the mean ADC decreased to  $0.800 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.675 \times 10^{-3}$ - $0.925 \times 10^{-3}$  mm<sup>2</sup>/s) at the 3<sup>rd</sup> week and  $0.766 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.659 \times 10^{-3}$ - $0.872 \times 10^{-3}$  mm<sup>2</sup>/s) at the 4<sup>th</sup> week. ADC increased again at the 5<sup>th</sup> week to  $0.839 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.702 \times 10^{-3}$ - $0.976 \times 10^{-3}$  mm<sup>2</sup>/s). We also observed a significant increase in the mean ADC value at the 2<sup>nd</sup> (*P* = 0.004) and 5<sup>th</sup> week (*P* = 0.033) during treatment relative to the values prior to treatment. The mean observed ADC values and *P* values are shown in Table 3.

### Tumor ADC for the prediction of treatment response

We compared the tumor ADC values of the responder and non-responder groups to predict the treatment response based on T downstage and TRG criteria.

Downstaging of the tumor was observed for 8 of the 15 patients (53.3%). The ADC values at the 5<sup>th</sup> week during treatment increased for 6/8 patients with T downstaging and increased for 5/7 patients without T downstaging relative to the mean tumor ADC values before treatment. The mean observed ADC values for patients with and without T downstage are shown in Table 4. The difference between these two groups with respect to the mean ADC values measured at the six timepoints did not reach significance.

For the eight patients with tumor downstaging, there was a significant increase (*P* = 0.011) in the mean tumor ADC at the 2<sup>nd</sup> week of treatment relative to the ADC before treatment, whereas for the seven patients without tumor downstaging, there was no significant change in the ADC at any timepoint during treatment relative to the ADC values before treatment (Figure 3A, Table 3).

Good regression (TRG 3-4) was observed in 9 of the 15 patients (60%), and poor regression (TRG 0-2) was observed in 6 patients (40%). The ADC values at the 5<sup>th</sup> week during treatment were increased in 7/9 patients with good regression and 5/6 patients with poor regression relative to the mean tumor ADC values before treatment. The mean observed ADC values in patients with good regression and poor regression are shown in Table 4. Before treatment and at the 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> week during treatment, significant differences in the mean ADC values between the two groups were obtained.

For the nine patients with good regression, there

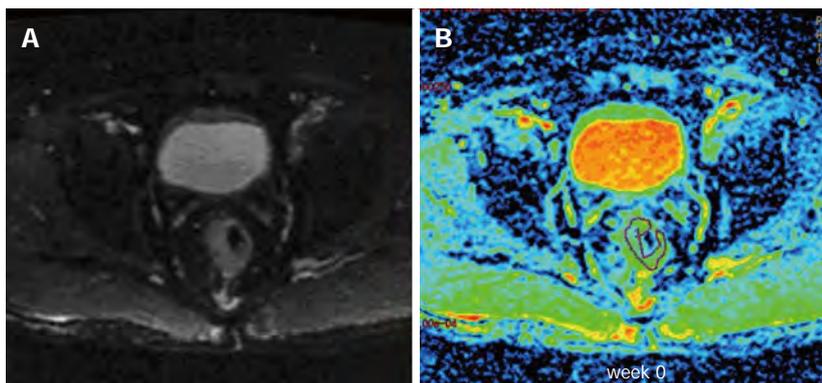
**Table 2** Apparent diffusion coefficient values at six measurement times

No.	Apparent diffusion coefficient values ( $\times 10^{-3} \text{ mm}^2/\text{s}$ )					
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
1	0.723	0.743	0.893	0.756	0.606	0.793
2	0.583	0.458	0.786	0.385	0.540	0.793
3	0.752	0.883	0.655	0.711	0.683	0.772
4	0.883	0.887	0.887	0.945	0.923	0.972
5	0.995	0.853	0.995	0.950	0.832	1.120
6	0.813	0.965	1.133	0.964	0.893	0.686
7	0.518	0.416	0.998	0.539	0.527	0.473
8	0.659	0.747	0.825	0.858	0.631	0.746
9	0.814	0.791	0.809	0.894	0.821	0.798
10	0.628	0.637	0.625	0.703	0.784	0.930
11	0.562	0.575	0.806	0.742	0.677	0.515
12	0.616	0.595	0.834	0.850	0.907	1.050
13	0.851	0.825	0.865	- <sup>1</sup>	0.831	0.882
14	0.592	- <sup>1</sup>	0.734	0.622	0.574	0.605
15	1.255	1.435	1.420	1.282	1.256	1.450
95%CI	0.641-0.858	0.627-0.918	0.775-0.994	0.675-0.925	0.659-0.872	0.702-0.976

<sup>1</sup>No data due to measurement error.

**Table 3** Mean tumor apparent diffusion coefficient values and the *P* values for the comparisons with the pretreatment values

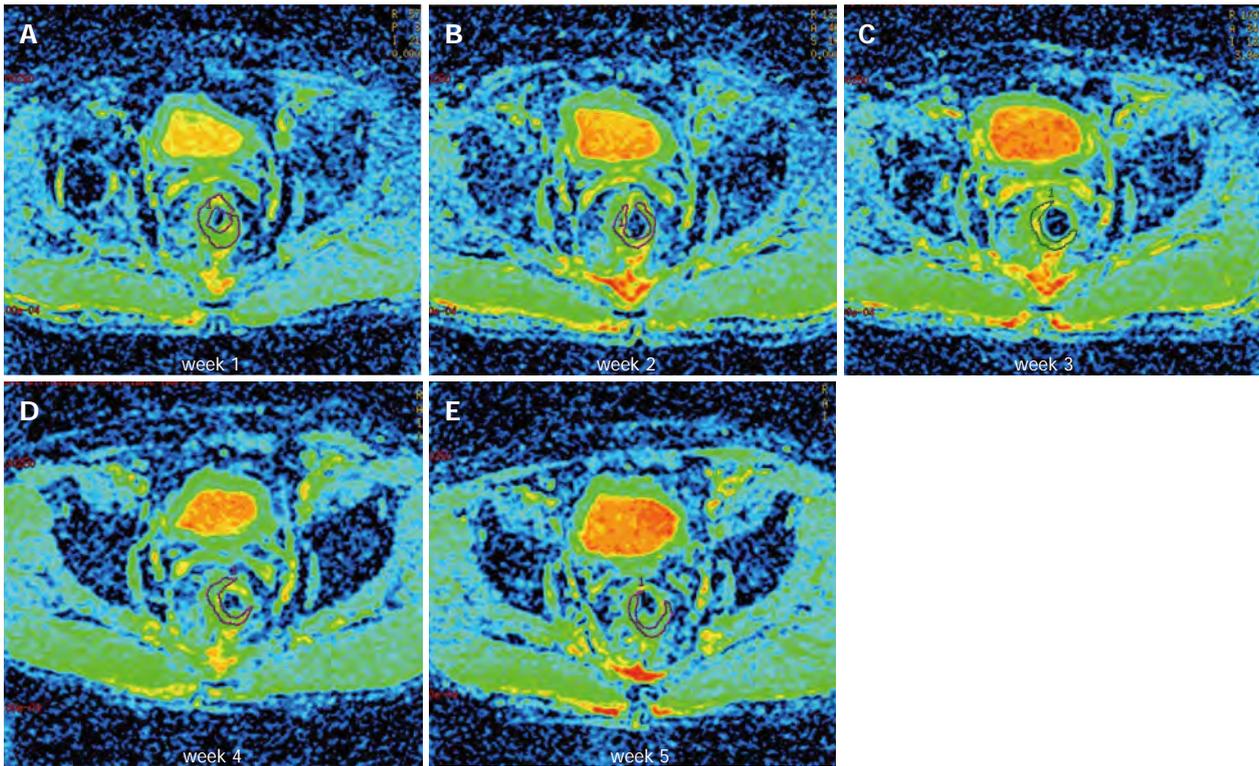
	Apparent diffusion coefficient values ( $\times 10^{-3} \text{ mm}^2/\text{s}$ )					
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
All ( <i>n</i> = 15)	0.749	0.772	0.884	0.800	0.766	0.839
<i>P</i> value	-	0.672	0.004	0.077	0.586	0.033
T downstage ( <i>n</i> = 8)	0.703	0.720	0.890	0.724	0.691	0.744
<i>P</i> value	-	0.964	0.011	0.406	0.578	0.284
No T downstage ( <i>n</i> = 7)	0.803	0.824	0.878	0.876	0.851	0.948
<i>P</i> value	-	0.617	0.185	0.117	0.430	0.074
Good regression ( <i>n</i> = 9)	0.659	0.671	0.852	0.696	0.674	0.714
<i>P</i> value	-	0.909	0.004	0.212	0.617	0.251
Poor regression ( <i>n</i> = 6)	0.886	0.907	0.933	0.938	0.904	1.027
<i>P</i> value	-	0.669	0.372	0.264	0.785	0.086



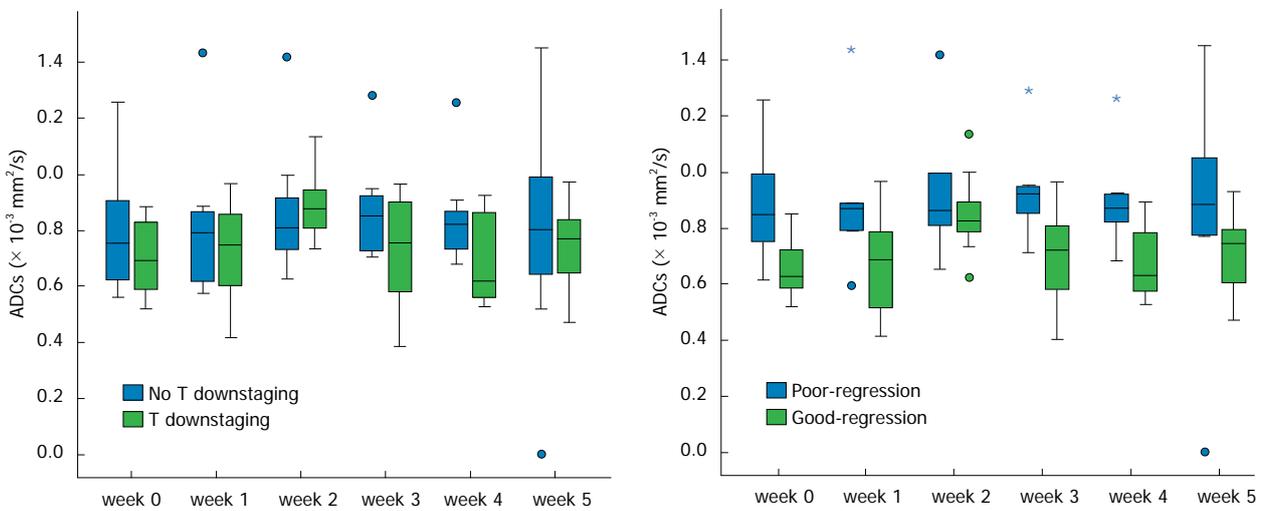
**Figure 1** Sample T2-weighted and diffusion-weighted images from a patient with locally advanced rectal cancer prior to treatment. A: T2-weighted transaxial image through the pelvis prior to treatment; B: Corresponding apparent diffusion coefficient map of the patient obtained from the diffusion-weighted images. A region of interest was drawn around the tumor.

was a significant increase ( $P = 0.004$ ) in the mean tumor ADC value at the 2<sup>nd</sup> week of treatment relative to the ADC values before treatment, whereas for the six patients with poor regression, no significant change in ADC was observed at any timepoint during treatment (Figure

3B, Table 3). The two patients with pCR demonstrated lower ADC ( $0.659 \times 10^{-3} \text{ mm}^2/\text{s}$  and  $0.813 \times 10^{-3} \text{ mm}^2/\text{s}$ ) before treatment but significantly increased tumor ADC ( $0.825 \times 10^{-3} \text{ mm}^2/\text{s}$  and  $1.133 \times 10^{-3} \text{ mm}^2/\text{s}$ ) at the 2<sup>nd</sup> week of treatment.



**Figure 2** Apparent diffusion coefficient maps of one patient obtained at constant intervals once weekly during the course of neoadjuvant treatment from the diffusion-weighted images. A-E: Axial apparent diffusion coefficient maps obtained during treatment (weeks 1-5). A region of interest was drawn around the tumor.



**Figure 3** Mean tumor apparent diffusion coefficient values in patients at six measurement points. A: With and without T downstaging; B: With good regression and poor regression. Cycle: Outlier; Star: Extreme value.

## DISCUSSION

The recent trend toward patient-tailored treatment for LARC has highlighted the need for a reliable method for the early assessment of treatment response. DWI-MRI may be a promising functional imaging tool for the prediction of treatment response. In our study, DW-MRI was investigated as a potential clinical tool to predict or assess the response of rectal tumors to neoadjuvant concurrent CRT at an early timepoint.

Our results show that CRT induced a significant increase in mean tumor ADC in LARC. Because the ADC values obtained from DWI measurements reflect tumor cellularity and anti-tumor treatment decreases tumor cellularity, CRT should increase the ADC value. The administration of CRT results in cell swelling, necrosis, and apoptotic cell death. When CRT is initiated, the ADC may rapidly decrease over several hours due to cell swelling, followed by an increase over several days concurrent with cell death. Increased ADC values have also been

**Table 4 Mean tumor apparent diffusion coefficient values and *P* values for the comparisons between groups**

Group	Apparent diffusion coefficient values ( $\times 10^{-3} \text{ mm}^2/\text{s}$ )					
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
T downstage ( $n = 8$ )	0.702	0.720	0.890	0.724	0.690	0.743
No T downstage ( $n = 7$ )	0.803	0.824	0.877	0.875	0.851	0.947
<i>P</i> value	0.339	0.463	0.909	0.202	0.108	0.114
Good regression ( $n = 9$ )	0.658	0.670	0.851	0.618	0.673	0.713
Poor regression ( $n = 6$ )	0.885	0.907	0.933	0.938	0.903	1.027
<i>P</i> value	0.021	0.081	0.452	0.032	0.016	0.010

correlated with tumor necrosis and reduced cell density<sup>[15]</sup>, and most studies have found an increase in ADC after CRT<sup>[12,16,17]</sup>. For example, Kim *et al.*<sup>[12]</sup> recently showed that neoadjuvant CRT caused a significant increase in the ADC values of 76 rectal cancer patients. In contrast, Hein *et al.*<sup>[18]</sup> reported a decrease in the ADC after CRT in all nine of their patients, and they attributed this result to intratumoral radiation-induced fibrosis and cytotoxic edema as well as to the method employed (ROI excluding apparent necrotic areas).

Our results indicate that the mean pre-CRT ADC was negatively correlated with tumor regression ( $P = 0.021$ ) but not with T downstaging ( $P = 0.339$ ). T downstaging and TRG criteria were used because these are common factors used for the evaluation of treatment responses<sup>[19,20]</sup>. The TRG was not completely concordant with T downstaging, and some studies have shown that the pretreatment ADC value is negatively correlated with treatment response in rectal cancer and other tumors<sup>[10,21-25]</sup>. Dzik-Jurasz *et al.*<sup>[10]</sup> found a strong negative correlation between the mean pretreatment tumor water ADC and the percent change in the size of the tumor after chemotherapy and chemoradiation in 2002. ADC values are generally higher for necrotic tumors than for solid or viable tumors<sup>[26]</sup>. Because necrotic areas in tumors are resistant to radiation, it may be hypothesized that tumors with necrotic areas, and thus high pretreatment ADC values, would have less favorable treatment responses. However, other studies have obtained different results; for example, several studies of rectal and other tumors found no correlation between the pretreatment ADC value and treatment response<sup>[11,27,28]</sup>, whereas another study found a positive correlation<sup>[12]</sup>. Several factors may explain these different correlations, such as small sample sizes, the use of different methods for calculating the ADC, and the use of different indicators for the evaluation of treatment response.

A substantial change in the mean ADC value at the 2<sup>nd</sup> week of CRT predicted the tumor response of LARC in our study. Most studies have assumed that CRT decreases tumor cellularity and results in a substantial change in the ADC value<sup>[18,26]</sup>. Although decreasing tumor cellularity will lead to a reduction in tumor size, this reduction is typically observed 3 wk or more after the start of CRT<sup>[29,30]</sup>. Thus, a more rapid evaluation or prediction of treatment response would be clinically useful. We

found a significant increase in the mean ADC at the 2<sup>nd</sup> week in the T downstage ( $P = 0.011$ ) and good regression ( $P = 0.004$ ) groups but not in the groups of patients without T downstaging and with poor regression. We believe that the significant increase in the mean ADC at the 2<sup>nd</sup> week of treatment was correlated with tumor necrosis and apoptosis, which reduce cell density, after the start of therapy. Similar results have been obtained in several other studies. For example, one study examined the ADC data of nine patients with LARC, and a significant change in the mean ADC starting at week 2 of CRT was observed<sup>[18]</sup>. In another study focused on the early detection of responses to CRT in cervical cancer, the changes in the ADC value after 2 wk of therapy were also significantly correlated with the treatment response<sup>[27]</sup>.

There are several limitations of our study. First, the study sample size was small. Second, the sample slice with the largest tumor extension was selected to determine the ADC value, and the use of this slice may not have adequately captured the heterogeneity of the tumor. Third, the ROIs were drawn manually, and this process may have influenced the ADC value and introduced subjectivity. The reason the ROIs were drawn manually by a single experienced radiologist was to obtain more uniform and stable ADC values.

Our study and several previous studies highlight the value of DW-MRI as a predictive tool for the response of rectal cancer to chemoradiation. However, there are some difficulties associated with incorporating DW-MRI into routine clinical practice. The reproducibility of DWI has been insufficiently investigated, and the cut-off values used to determine treatment response vary between treatments and ADC measurement techniques. Thus, a standardized guideline to predict or assess treatment response is needed before DWI can be implemented in clinical practice.

In this study, the tumor ADC values changed during the course of neoadjuvant chemoradiation. The pretreatment tumor ADC value was negatively correlated with tumor regression after chemoradiation for the treatment of LARC, and the ADC value at the 2<sup>nd</sup> week of therapy was significantly correlated with the tumor response. Our results indicate that DW-MRI may be a valuable clinical tool to help predict or assess the responses of rectal tumors to neoadjuvant concurrent chemoradiation at an early timepoint.

## COMMENTS

**Background**

Neoadjuvant (chemo)radiation followed by surgery has become the standard treatment for locally advanced rectal cancer (LARC). However, approximately 20%-30% of patients do not benefit from this neoadjuvant treatment due to radioresistance of the tumor. Functional non-invasive diffusion-weighted magnetic resonance imaging (DW-MRI) studies are increasingly used to predict response to cancer therapy, but definitive evidence is limited, especially for patients with rectal cancer treated with neoadjuvant chemoradiation therapy (CRT).

**Research frontiers**

DW-MRI is a non-invasive functional MRI technique. To date, published data on the value of DW-MRI as a predictive tool for assessing responses to anti-cancer treatment in patients with rectal cancer are scarce and conflicting.

**Innovations and breakthroughs**

The authors found that CRT induced a significant increase in the mean apparent diffusion coefficient (ADC) value of LARC. The pretreatment tumor ADC was negatively correlated with tumor regression after CRT for the treatment of LARC, and the ADC value at the 2<sup>nd</sup> week of therapy was significantly correlated with the tumor response.

**Applications**

The results of this study suggest that DW-MRI may be a valuable clinical tool to help predict or assess the responses of rectal tumors to neoadjuvant concurrent CRT at an early timepoint.

**Terminology**

DW-MRI is a non-invasive functional MRI technique that provides information by measuring water proton mobility in tissues. ADC values can be calculated from DWI measurements according to the impediment to free diffusion of water molecules in a single voxel due to restricting barriers such as membranes, macromolecules, and fibers inside different tissue compartments.

**Peer review**

This is an interesting study that investigates the use of DW-MRI as a predictor of the tumor response in 15 patients with rectal cancer undergoing CRT therapy by measuring the tumor ADC. This is an emerging field in which new knowledge is needed, and this study, despite its limits, provides novel information that may help to settle the current debate about the utility of DW-MRI as a predictive tool for the response to anti-cancer treatment.

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## Dual-sided composite mesh repair of hiatal hernia: Our experience and a review of the Chinese literature

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### Abstract

**AIM:** To summarize our experience in the application of Crurasoft® for antireflux surgery and hiatal hernia (HH) repair and to introduce the work of Chinese doctors on this topic.

**METHODS:** Twenty-one patients underwent HH repair with Crurasoft® reinforcement. Gastroesophageal reflux disease (GERD) and HH-related symptoms including heartburn, regurgitation, chest pain, dysphagia, and abdominal pain were evaluated preoperatively and 6 mo postoperatively. A patient survey was conducted by phone by one of the authors. Patients were asked about "recurrent reflux or heartburn" and "dysphagia". An internet-based Chinese literature search in this field was also performed. Data extracted from each study included: number of patients treated, hernia size, hiatorrhaphy, antireflux surgery, follow-up period, recurrence rate, and complications (especially dysphagia).

**RESULTS:** There were 8 type I, 10 type II and 3 type III HHs in this group. Mean operative time was 119.29 min (range 80-175 min). Intraoperatively, length and width of the hiatal orifice were measured, ( $4.33 \pm 0.84$  and  $2.85 \pm 0.85$  cm, respectively). Thirteen and eight Nissen and Toupet funduplications were performed, respectively. The intraoperative complication rate was 9.52%. Despite dysphagia, GERD-related symptoms improved significantly compared with those before surgery. The recurrence rate was 0% during the 6-mo follow-up period, and long-term follow-up disclosed a recurrence rate of 4.76% with a mean period of 16.28 mo. Eight patients developed new-onset dysphagia. The Chinese literature review identified 12 papers with 213 patients. The overall recurrence rate was 1.88%. There was no esophageal erosion and the rate of dysphagia ranged from 0% to 24%.

**CONCLUSION:** The use of Crurasoft® mesh for HH repair results in satisfactory symptom control with a low recurrence rate. Postoperative dysphagia continues to be an issue, and requires more research to reduce its incidence.

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**Key words:** Hiatal hernia; Gastroesophageal reflux disease; Anti-reflux surgery; Mesh; Prosthetic

**Core tip:** With a focus on the mesh fixation technique, the application of Crurasoft® for antireflux surgery and hiatal hernia repair achieved satisfactory outcome. The recurrence rate was 0% during the 6-mo follow-up period, and long-term follow-up disclosed a recurrence rate of 4.76% with a mean period of 16.28 mo. Eight patients developed new-onset dysphagia and this gradually resolved without difficulty in swallowing solid food in 6 patients. The Chinese literature review identified 12 papers with 213 patients. The overall recurrence rate was 1.88%. There was no esophageal erosion and

the rate of dysphagia ranged from 0% to 24%.

Zhang W, Tang W, Shan CX, Liu S, Jiang ZG, Jiang DZ, Zheng XM, Qiu M. Dual-sided composite mesh repair of hiatal hernia: Our experience and a review of the Chinese literature. *World J Gastroenterol* 2013; 19(33): 5528-5533 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5528.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5528>

## INTRODUCTION

Laparoscopic fundoplication is a safe and effective alternative to long-term medical treatment for patients with gastroesophageal reflux disease (GERD) and hiatal hernia (HH)<sup>[1]</sup>. Cryoplasty is considered to be an essential part of antireflux surgery<sup>[2]</sup>. Possible reasons for failed laparoscopic antireflux surgery and disruption of HH repair are lateral tension following simple hiatal closure, or poor character of the crural musculature. The use of a mesh, either by reducing tension or reinforcing the crural musculature, is associated with a significantly lower recurrence rate<sup>[1-5]</sup>.

Despite this, most concerns are focused on mesh-related complications (including intraluminal erosion, fibrosis, and esophageal stenosis)<sup>[3,6]</sup>. Although few mesh-related complications at the hiatus have been reported, anecdotal observations suggest that this complication may be more common<sup>[7]</sup>. Moreover, surgery to manage these complications is complex and may require esophagectomy or gastrectomy<sup>[3]</sup>. For these reasons, many surgeons avoid the use of synthetic mesh in HH repair<sup>[8]</sup>.

The ideal mesh generates adhesion to the diaphragmatic surface and not the visceral side<sup>[4]</sup>. "V" shaped composite polytetrafluoroethylene (PTFE) and expanded polytetrafluoroethylene (ePTFE) prostheses (dual-sided composite mesh, Crurasoft<sup>®</sup>) have some of these features. PTFE encourages ingrowth of host tissue from the underlying crura, producing local fibrosis and a more uniform mesh-tissue complex. ePTFE was thought to have a benign behavior as opposed to hollow viscera<sup>[9]</sup>, with encapsulation of the material and neomesothelialization of the exposed abdominal surface, thus becoming isolated from the esophagus and stomach<sup>[3]</sup>. That is, dual-sided mesh has the merits of prosthetic mesh and may avoid possible major complications. Chilintseva *et al*<sup>[10]</sup> reported the preliminary results of the use of this dual-sided prosthesis for large HH repairs, demonstrating satisfactory results. Although there was no erosion of the esophagus or stomach, severe periprosthetic fibrosis resulted in postoperative dysphagia in two patients, requiring reoperation. The authors proposed that positioning the mesh with care should be emphasized<sup>[10]</sup>.

To reduce postoperative dysphagia, some propose that space should be allowed between the esophagus and the mesh<sup>[11]</sup>. We summarize our experience in the ap-

plication of Crurasoft<sup>®</sup> for antireflux surgery and HH repair, focusing on whether a reduction in postoperative complications, especially erosion and dysphagia, can be achieved if technical attention to mesh fixation is applied. Moreover, Crurasoft<sup>®</sup> is the most commonly used prosthetic mesh in China. We also analyzed and introduced the work of Chinese doctors on this topic, as the Chinese language is still an obstacle for academic communication.

## MATERIALS AND METHODS

### Patients

From May 2010 to July 2012, 48 patients underwent surgery for pH-proven symptomatic GERD with HH in our institution. Of these, 21 patients (14 male, 7 female) underwent hiatal repair with an onlay Crurasoft<sup>®</sup> mesh reinforcement and were enrolled in this retrospective analysis. The indication for mesh implantation included a HH length longer than 3 cm, obesity, and weak hiatus tissue.

### Symptom evaluation

GERD and HH related symptoms including heartburn, regurgitation, chest pain, dysphagia, and abdominal pain were evaluated preoperatively and 6 mo postoperatively. The severity of symptoms was evaluated using a scaled 0-10 visual analog score, as previously described in the literature<sup>[12]</sup>.

### Preoperative work-up

Preoperative barium contrast swallowing or a computed tomography scan was used to evaluate the type and size of the HH. The presence and severity of esophagitis was confirmed by upper endoscopy. pH monitoring (24-h) and esophageal manometry were performed in all patients to evaluate lower esophageal sphincter function and esophageal motility.

### Surgical technique

Five trocars were used during laparoscopic surgery. The stomach was first reduced into the abdomen, followed by mobilization of the distal esophagus with at least 3 cm of intraabdominal esophagus restored to the abdominal cavity. All patients underwent primary closure of the hiatus with between 2 and 5 nonabsorbable sutures for posterior Cryoplasty, depending on the size of the hiatus defect (Figure 1). Additional anterior Cryoplasty was also performed if the defect was wide. A V-shaped dual-sided composite mesh (Crurasoft<sup>®</sup>, Composix mesh, CR Bard, Cranston, United States) was used to reinforce the primary repair, with the PTFE side facing the diaphragm (Figure 2). The lower of the two arms was positioned about 2-3 mm below the first stitch, and fixed with staples (EMS, Johnson and Johnson). Additional staples were applied to secure the mesh to the right and left crura and flatten it. The small ePTFE "tongue" was placed to protect the posterior esophageal wall from contacting the PTFE margin. After closing the hiatus, a fundoplication (Nissen/Toupet) was performed.

**Table 1** Classification of hiatal hernia

Type	Description
I	Sliding hernia with the GEJ above the diaphragm
II	Paraesophageal hiatus hernia. A part of the stomach herniates through the hiatus and lies beside the esophagus, without movement of the GEJ
III	Combined hernia. The combination of type I and II
IV	A large defect in the hiatus, allowing other organs to enter the hernia sac

GEJ: Gastroesophageal junction.

**Table 2** Baseline characteristics of the patients who underwent laparoscopic antireflux surgery

Item	Value
Age (yr)	53.81 ± 13.76 (21-75)
Body mass index (kg/m <sup>2</sup> )	28.95 ± 3.11 (21-35)
Hiatal hernia length (cm)	4.33 ± 0.84 (3.1-6.3)
Hiatal hernia width (cm)	2.85 ± 0.85 (1.8-5.4)
DeMeester score	50.30 ± 27.73 (12.7-112.7)
Surgery duration (min)	119.29 ± 23.84 (80-175)
Postoperative stay (d)	4.71 ± 0.85 (4-7)

Data are expressed as absolute mean ± SD (range).

### Intraoperative data

Operative duration and size of HH (length and width), were recorded.

### Phone questionnaire

In January 2013, a patient survey was conducted by phone by one of the authors. Patients were asked about “recurrent reflux or heartburn” and “dysphagia.” Dysphagia was defined as new-onset difficulty in swallowing; severity (mild/severe) and duration of symptoms (temporary/permanent, duration shorter than 6 mo was defined as temporary) were also surveyed. Patients were also asked about “whether you are satisfied with the outcome of surgery”.

### Data sources and study selection

An internet-based Chinese literature search was performed using the Chinese Medical Literature database (Chongqing VIP) between January 2000 and December 2012. The key words “hiatal hernia”, “GERD”, and “mesh” were used in all possible combinations to identify relevant articles. If data appeared appropriate for analysis, the abstract and full article were retrieved for in-depth review. All reference lists in the papers were manually searched for relevant articles. Inclusion criteria were: (1) antireflux surgery with HH repair using Crurasoft® composite mesh; (2) reports described surgical technique details; and (3) reports documented outcome of recurrence and follow-up data. The literature search, study selection, and data extraction were performed by two independent authors. Data extracted from each study included: number of patients treated, hernia size, hiatorrhaphy, antireflux surgery, follow-up period, recurrence rate, and

**Table 3** Symptom evaluation before and after surgery

	Before surgery	After surgery	P value
Heartburn	5.33 ± 1.65	2.14 ± 1.74	0
Regurgitation	5.00 ± 1.64	1.95 ± 1.16	0
Chest pain	3.62 ± 1.99	1.29 ± 1.15	0
Dysphagia	2.57 ± 1.66	1.62 ± 1.86	0.087
Abdominal pain	2.33 ± 1.28	1.38 ± 1.12	0.014

complications (especially dysphagia).

### Ethical approval of the study protocol

All patients were informed about the study protocol. Written consent for the investigation in accordance with the ethical guidelines of Changzheng Hospital was obtained.

### Statistical analysis

The student's *t* test and Pearson  $\chi^2$  test were used to compare means and categorical variables, respectively. A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### Perioperative data and 30-d complications

Classification of HH is listed in Table 1. There were 8 type I, 10 type II, and 3 type III HHs in this group. Mean operative time was 119.29 min (range 80-175 min). Intraoperatively, both the length and width of the hiatal orifice were measured, (4.33 ± 0.84 and 2.85 ± 0.85 cm, respectively; Table 2). Thirteen and 8 Nissen and Toupet funduplications were performed, respectively.

There was no mortality and no conversion to open surgery. The intraoperative complication rate was 9.52% (one spleen capsular laceration and 1 pneumothorax, all repaired laparoscopically without sequelae). Eight patients complained of new-onset dysphagia with difficulty eating solid food. No early reoperation or intervention (*e.g.*, endoscopic dilatation) was required. The median length of postoperative hospital stay was 5 (range 4-7) d.

### Symptomatic improvement

Preoperative and postoperative symptoms of GERD and HH were compared. As listed in Table 3, all relevant symptoms (except for dysphagia) improved significantly. 10 patients agreed to have a barium meal; no recurrence was demonstrated.

### Long-term complications

One patient was lost to follow-up. The mean follow-up period was 16.28 (6-32) mo. The overall satisfaction rate was 85.71% (18/21). One patient had a recurrence confirmed at the 8 mo postoperative visit, with the major complaint being dysphagia, different from her preoperative symptoms of heartburn. Barium meal examination showed a type II paraesophageal hernia. In a review of her history, she developed dysphagia at postoperative month 3, following an episode of severe vomiting. This

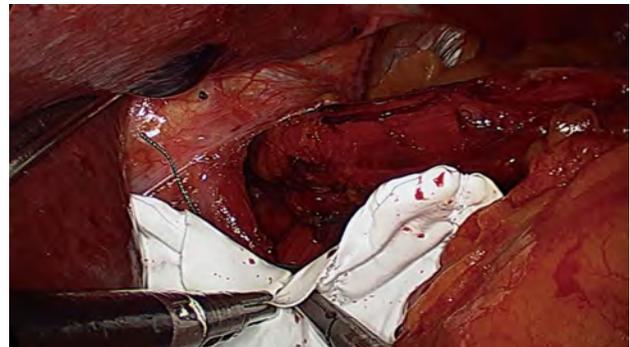
**Table 4** Chinese literature on the use of dual-sided mesh for hiatal hernia repair

Author	Patients	Hernia size	Hiatorrhaphy	Antireflux surgery	Follow-up (mo)	Recurrence rate	Complications
Chu <i>et al</i> <sup>[13]</sup>	12	III (8), IV (4)	Yes	Nissen	12-60	0/12	-
Wang <i>et al</i> <sup>[14]</sup>	15	I (6), II (7), III (2)	Yes	Toupet	Median 18	0/15	1 dysphagia, 2 PPI treatment
Tai <i>et al</i> <sup>[15]</sup>	21	I (9), II (4), III (6), IV (2)	Yes	Toupet	1-16	0/21	3 dysphagia
Ji <i>et al</i> <sup>[16]</sup>	7	-	Yes	Nissen	6-24	0/7	-
Ma <i>et al</i> <sup>[17]</sup>	40	I 1 (3), II (4), III (15), IV (8)	Yes	Toupet/Dor	3-25	0/40	6 dysphagia
Zhao <i>et al</i> <sup>[18]</sup>	25	All > 6 cm	Yes	Nissen/Toupet/Dor	3-35	1 (1)/25 <sup>1</sup>	6 dysphagia, 1 PPI treatment
Xu <i>et al</i> <sup>[19]</sup>	3	13-18 cm	Yes	Toupet	6-12	0/3	-
Zhang <i>et al</i> <sup>[20]</sup>	21	I 1 (4), II (5), III (2)	Yes	Toupet	6-36	0/21	-
Zou <i>et al</i> <sup>[21]</sup>	20	All > 6 cm	Yes	Dor	> 12	2 (5)/20 <sup>1</sup>	-
Yao <i>et al</i> <sup>[22]</sup>	33	I (5), II (23), III (5)	Yes	Nissen	> 12	1 (10)/33 <sup>1</sup>	3 dysphagia, 1 gastric retention
Li <i>et al</i> <sup>[23]</sup>	4	-	Yes	Nissen/Toupet	1-36	0/4	-
Fei <i>et al</i> <sup>[24]</sup>	12	< 5 cm (10), > 5 cm (2)	Yes	Nissen	12	0/12	1 dysphagia

<sup>1</sup>Anatomic recurrence (symptomatic recurrence)/total patient number. PPI: Proton pump inhibitor.



**Figure 1** Primary closure of the hiatus with nonabsorbable stitches for posterior Cryoplasty.



**Figure 2** Lower margin of the two arms was positioned about 2-3 mm below the first stitch.

patient scored the outcome of surgery as dissatisfactory and was reluctant to undergo reoperation. The other two patients presented with recurrence of heartburn or regurgitation 4 and 7 mo following surgery, respectively. In these cases, barium swallowing failed to detect HH recurrence. Both patients underwent Nissen fundoplication without symptoms of dysphagia.

Of the 8 patients who complained of postoperative dysphagia, this gradually resolved without difficulty in swallowing solid food in 6 patients. The average period to resolution of dysphagia was 5.2 (range 4-7) mo. The remaining 2 patients complained of mild to moderate dysphagia, unabated even at the final phone call contact (11 and 19 mo postoperatively, respectively). One patient was confirmed to have a slight stricture at the level of the hiatus, for which dilatation achieved slight resolution. The other patient refused further workup and intervention.

### Chinese literature review

Our literature search identified 24 articles for review. Twelve papers fulfilled the inclusion criteria, with a total of 213 patients included in the final analysis. Reasons for exclusion were: no follow-up data ( $n = 8$ ), pediatric surgery ( $n = 1$ ), review ( $n = 1$ ), and overlapping study populations ( $n = 2$ ) (Table 4)<sup>[13-24]</sup>. All surgery involved hiatoplasty and fundoplication other than gastropexy. There were only 3 randomized controlled trials. Fei *et*

*al*<sup>[24]</sup> concluded that reinforcement of HH repair with Crurasoft<sup>®</sup> significantly improved HH-related symptoms. Zou *et al*<sup>[21]</sup> confirmed that the use of Crurasoft<sup>®</sup> significantly reduced recurrence from 36.4% with simple closure to 10%, with a follow-up period greater than 1 year. Yao *et al*<sup>[22]</sup> reported that there was 1 hernia recurrence following mesh placement in the 1-year follow-up period, compared with 3 cases in the simple Cryoplasty group. However, this difference was not statistically significant ( $P = 0.300$ ). Recurrence rates varied from 0% to 25%. This disparity might be due to differences in the definition of recurrence. Some authors define recurrence as symptoms, without radiological confirmation. The highest anatomic recurrence was reported by Zou *et al*<sup>[21]</sup> (10%), where all the HH in that cohort were larger than 6 cm. The overall anatomic recurrence rate was 1.88% (4/213). There were no cases of esophageal or stomach erosion. Postoperative dysphagia varied from 0% to 24% (median 6.67%).

### DISCUSSION

A survey on the use of mesh for HH repair by members of the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) showed that 33% preferred nonabsorbable to absorbable mesh<sup>[25]</sup>. This reflects the fact that prosthetic mesh has the advantage of reducing HH recurrence; biomaterial tends to be associated with

failure<sup>[9]</sup>. On the other hand, concerns still exist regarding mesh-related complications, including erosion, stricture, and fibrosis. Thus, there may be a trade-off in the choice of mesh repair for HH: permanent mesh risks erosion, while biologic mesh risks recurrence<sup>[9]</sup>.

Crurasoft<sup>®</sup> has the advantages of permanent mesh, while reducing mesh-related complications. Chilintseva *et al*<sup>[10]</sup> reported 38 cases who underwent HH repair using Crurasoft<sup>®</sup>, with no recurrences. Priego *et al*<sup>[26]</sup> concluded that Crurasoft<sup>®</sup>-reinforced hiatoplasty reduced HH recurrence in patients with large hiatal defects (larger than 5 cm), similar to that in patients with smaller hiatal defects (2% *vs* 2.1%). Granderath *et al*<sup>[27]</sup> selected a tailoring strategy for HH repair according to hiatal surface area (HAS). Those with HAS larger than 8 cm<sup>2</sup> underwent Crurasoft<sup>®</sup> placement in a tension-free, posterior onlay fashion. During a mean follow-up period of 6.3 mo, only 1 patient (1.8%) developed postoperative partial intrathoracic wrap migration. In the Chinese literature review, recurrence was between 0% and 10% (Table 3). The highest recurrence rate (2/20, 10%) was reported by Zou *et al*<sup>[21]</sup>, in whose series all HH were large, with orifices larger than 6 cm or herniation of more than half of the stomach. In our study cohort, we found a type II HH anatomic recurrence. Paraesophageal herniation is a complication that occurs in the immediate postoperative period following laparoscopic antireflux surgery, with an incidence of up to 7%. Vomiting in the early postoperative period, which occurred in this patient, has been identified as a risk factor for recurrence<sup>[3]</sup>. Violent diaphragmatic movements might also dislodge the mesh if fixation is inadequate<sup>[7]</sup>. Sufficient fixation of the mesh and avoidance of lifting or straining have been advocated to reduce this complication.

Two patients suffered symptomatic recurrence without any proof of anatomic recurrence. Both patients underwent Nissen fundoplication and the barium meal examination showed an intact wrap. A possible explanation for this could be poor correlation between postoperative symptoms and actual reflux<sup>[28]</sup>. Both patients presented with heartburn and acid regurgitation, the cardinal symptoms of GERD. However, these symptoms have a low specificity and sensitivity for the actual diagnosis of GERD. One patient agreed to resume manometry and pH monitoring, and all data indicated an improvement compared with that before surgery. As postoperative GERD symptoms actually indicate acid reflux in only 30% of patients and are not even accurate to rule out acid reflux in patients who are completely free of symptoms after surgery, Khajanchee *et al*<sup>[28]</sup> insisted that surgeons should explain the presence of symptomatic recurrence cautiously and that objective testing should be introduced to determine the actual cause.

In a collection of case reports pertaining to mesh complications after prosthetic hiatoplasty with special emphasis on mesh erosion, Stadlhuber *et al*<sup>[7]</sup> identified 17 cases of intraluminal erosion, involving not only different mesh material (polypropylene, PTFE, and biomaterial), but also different mesh configurations (keyhole, horse-

shoe and heart shaped). No apparent relationship between these parameters and mesh erosion was observed, thus the technique for mesh fixation was questioned. Fixation techniques such as the proximity of placement of the mesh at the esophagus are important factors in the development of postoperative complications. The edge of the mesh may “cheese wire” its way into the esophagus if it touches the esophagus or if shrinkage occurs. It is also possible that the mesh can migrate if fixation is insufficient, or traumatic events such as vomiting or repeated coughing may dislodge the mesh, causing it to be apposed to the esophageal wall, leading to erosion or stricture<sup>[7]</sup>. Use of Crurasoft<sup>®</sup> cannot completely eliminate this complication. In one case report, total migration of Crurasoft<sup>®</sup> into the stomach was detected by endoscopy 2 years after repeat fundoplication<sup>[29]</sup>. Both our series and a review of the Chinese literature failed to disclose any cases of this complication. However, a case of erosion was discussed at a conference without confirmation of its exact source and details (personal communication). Thus, the exact incidence of this complication may be underestimated<sup>[7,11]</sup>.

As opposed to erosion, esophageal stricture due to fibrosis associated with the prosthesis, may be a more common complication. Although ePTFE is less fibrogenic and is designed to prevent contact between the mesh and viscera, severe fibrosis enveloping the mesh can develop, leading to stricture refractory even to endoscopic dilatation<sup>[10]</sup>. Even though Wassenaar’s recommendations to maintain a 2-3 mm distance between the mesh and esophagus were followed, postoperative dysphagia cannot be completely eliminated (38.10% in our cohort). Only 2 patients had permanent symptoms, due to stricture at the hiatus and not the fundoplication itself (confirmed radiographically). Fortunately, most of these patients presented with mild dysphagia, which resolved within the first postoperative year, and did not require reoperation.

In conclusion, the use of Crurasoft<sup>®</sup> mesh for HH repair results in satisfactory symptom control with a low recurrence rate. Postoperative dysphagia continues to be an issue, and requires more research to reduce its incidence.

## COMMENTS

### Background

Cryoplasty is considered to be an essential part of antireflux surgery and use of a mesh, either by reducing tension or reinforcing the crural musculature, is associated with a significantly lower recurrence rate. Despite this, most concerns are focused on mesh-related complications.

### Research frontiers

Prosthetic mesh-related complications, including intraluminal erosion, fibrosis, and esophageal stenosis may be more common than reported. Moreover, surgery to manage these complications is complex and may require esophagectomy or gastrectomy. The ideal mesh generates adhesion to the diaphragmatic surface and not the visceral side.

### Innovations and breakthroughs

“V” shaped composite polytetrafluoroethylene (PTFE) and expanded polytetrafluoroethylene (ePTFE) prostheses (dual-sided composite mesh, Crurasoft<sup>®</sup>) might be an ideal mesh. PTFE encourages ingrowth of host tissue from the underlying crura, producing local fibrosis and a more uniform mesh-tissue complex. ePTFE was thought to have a benign behavior as opposed to hollow viscera,

with encapsulation of the material and neomesothelialization of the abdominal exposed surface, thus becoming isolated from the esophagus and stomach.

### Applications

Crurasoft® is the most commonly used prosthetic mesh in China. The authors summarize their experience in the application of Crurasoft® for antireflux surgery, focusing on whether reduction of postoperative complications, especially erosion and dysphagia, can be achieved if technical attention to mesh fixation is applied.

### Peer review

Clinical study and review by the authors demonstrate the benefit of the mesh Crurasoft in surgical therapy of hiatal hernia. The study is of clinical interest.

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## Proposal of new classification for postoperative patients with hepatocellular carcinoma based on tumor growth characteristics

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### Abstract

**AIM:** To propose an appropriate staging system for hepatocellular carcinoma (HCC) classification.

**METHODS:** Here, 288 in-patients with HCC were studied and divided into three groups: those with expansive growth, invasive growth (including satellite nodules, nodule fusions and direct tumor invasion of adjacent organs), or disseminative growth (including vascular involvement, regional lymph node metastasis and distant metastasis). A survival analysis was performed using a Kaplan-Meier analysis, and prognostic factors for overall survival were determined by the Cox proportional hazards regression model.

**RESULTS:** The overall survival (OS) of patients with invasive tumor growth was shorter than that of patients with expansive tumor growth ( $27.796 \pm 3.730$  and  $57.398 \pm 4.873$  mo, respectively,  $P < 0.001$ ). No significant difference in survival was observed between

patients with vascular involvement and patients with regional lymph node metastasis ( $21.667 \pm 4.773$  and  $14.619 \pm 2.456$  mo, respectively,  $P = 0.801$ ). The OS of patients with distant metastasis ( $6.417 \pm 1.395$  mo) was shorter than that of the other groups ( $P < 0.001$ ). No significant difference in survival was observed between patients with expansive tumor growth and vascular and/or regional lymph node involvement and patients with invasive tumor growth and no vascular and/or lymph node involvement ( $25.762 \pm 7.024$ ,  $21.200 \pm 7.794$  and  $39.533 \pm 5.840$  mo, respectively;  $P = 0.871$ ,  $0.307$  and  $0.563$ , respectively).

**CONCLUSION:** These data led to the proposal of a new staging system: the Expansive-Invasive-Disseminative growth staging classification.

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**Key words:** Hepatocellular carcinoma; Lymph node; Metastasis; Invasive growth; Staging system classification

**Core tip:** A number of staging systems were designed for all of hepatocellular carcinoma (HCC) patients based on some character of tumor, such as tumor size, vascular invasion, regional lymph node metastasis and extra-hepatic spread. But those systems failed to adequately stratify HCC patients with respect to prognosis. In our study, we explore an appropriate staging system for resectable patients with HCC based on tumor's growth characteristics, the Expansive-Invasive-Disseminative growth staging classification, which is a simple and efficacious prognostic model for postoperative patients with HCC.

Zhu CH, Liu XH, Cao R, Wu XZ. Proposal of new classification for postoperative patients with hepatocellular carcinoma based on tumor growth characteristics. *World J Gastroenterol* 2013; 19(33): 5534-5541 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

A staging system has been widely used for malignant diseases to stratify patients into comparable groups to predict patients' long-term outcomes. The American Joint Committee on Cancer (AJCC) uses the tumor-node-metastasis (TNM) system as a staging system for many malignant diseases to predict prognosis. Nevertheless, the TNM system fails to adequately stratify hepatocellular carcinoma (HCC) patients with respect to prognosis. In fact, prognosis of patients with cirrhosis and HCC depends on both residual liver function and tumor characteristics. Staging systems that include liver function status were first proposed by Okuda *et al*<sup>[1]</sup>. In 1998, investigators from the Cancer of the Liver Italian Program (CLIP) proposed the CLIP score that is based on Child-Pugh stage, tumor morphology and extension, alpha-fetoprotein (AFP) level, and portal vein thrombosis<sup>[2,3]</sup>. Although the CLIP score has good prognostic value in HCC patients, this score has some limitations when applied to patients with resectable HCC<sup>[4]</sup>. The Chinese University Prognostic Index for HCC was identified in 2002<sup>[5]</sup>. It combines the conventional TNM system with liver function and AFP. In 2003, the Japan Integrated Staging score was proposed by Kudo *et al*<sup>[4]</sup>. It is based on new adapted TNM system and Child-Pugh grading.

Some of the tumor characteristics of HCC include the tumor size, tumor number, invasive growth, vascular invasion, regional lymph node metastasis, and extrahepatic spread<sup>[6-17]</sup>. The TNM staging system is based on tumor characteristics, such as tumor size, vascular invasion, regional lymph node metastasis and extrahepatic spread. In 1999, the Barcelona Clinic Liver Cancer (BCLC) staging classification for HCC was proposed by Llovet *et al*<sup>[18]</sup> based on certain tumor characteristics. Cammà *et al*<sup>[19]</sup> reported that the overall predictive ability of BCLC, CLIP and French classification staging systems was unsatisfactory for patients with both cirrhosis and HCC and did not have uniform predictive results for treated patients and untreated patients. None of the scoring systems provided confident prediction of survival in individual patients. However, because the liver function of the most resectable patients is either an A or B score by Child-Pugh analysis, the TNM staging system provides an effective means of assessing the prognosis of patients following curative resection of HCC<sup>[11]</sup>. Unfortunately, the TNM system fails to include comprehensive characteristics of the tumor, especially the tumor's growth pattern. Thus, it is crucial to design a system to evaluate the effects of tumor characteristics on the clinical outcome of resectable patients with HCC.

In this study, 288 postoperative patients with HCC were studied and followed until August 2012. Patients with a C score from the Child-Pugh analysis were ex-

cluded to eliminate the effect of poor liver function on the long-term outcome. The purpose of the study was to explore an appropriate staging system for resectable patients with HCC based on the patient's tumor growth characteristics.

## MATERIALS AND METHODS

### Patients

Two hundred and eighty-eight in-patients who were diagnosed with HCC and underwent curative resection of HCC at Tianjin Medical University Cancer Institute and Hospital from March 1999 to July 2007 were included in this study and were followed until August 2012. Pathological testing for all patients was performed to confirm HCC. Contrast-enhanced computed tomography (CT), magnetic resonance imaging or positron emission tomography-CT was performed to confirm patients without metastatic disease. The patients' medical records were reviewed, and demographic, clinical and histological variables were derived from the medical records. The pT and pN status were identified based on the 7<sup>th</sup> edition of AJCC TNM classification. This study was approved by our institutional research review board.

### Statistical analysis

Overall survival (OS) curves were plotted by the Kaplan-Meier method and compared using the Log-rank test. The prognostic factors which showed the potential associations with OS were analyzed using a univariate analysis. They Cox proportional hazard model was used to find independent characteristic factors for survival time for the multivariate analysis from the univariate analyses. Statistical calculations were performed using SPSS (Version: 16.0, Chicago, United States).

## RESULTS

### Characteristics of patients

All of the patients with HCC had an A and B score by Child-Pugh analysis. The patients and tumor characteristics are summarized in Table 1. The median age of patients in this study was 54 years. In total, 119 patients had stage I, 22 patients had stage II, 24 patients had stage IIIA, 25 patients had stage IIIB, 66 patients had stage IIIC, and 21 patients had stage IVA HCC. Although most patients with stage IVB HCC were excluded from tumor resection, 11 patients at Tianjin Medical University Cancer Institute and Hospital in stage IVB had a resected primary tumor from March 1999 to July 2007. The OS and median survival time were  $45.704 \pm 3.380$  and  $20.000 \pm 2.314$  mo, respectively, for postoperative patients with HCC. Tumor size, tumor status, regional lymph node metastasis, distant metastasis, Child-Pugh score, AFP and tumor growth pattern were the factors affecting survival (Table 2).

### Survival analysis of invasive growth

Patients were divided into five groups based on tumor

**Table 1 Patients and tumor characteristics *n* (%)**

Characteristics	<i>n</i> = 288
Age (yr)	
≤ 60	208 (72.2)
> 60	80 (27.8)
Gender	
Female	49 (17.0)
Male	239 (83.0)
HBV infection	211 (75.36)
TNM stage	
I	119 (41.3)
II	22 (7.6)
III A	24 (8.3)
III B	25 (8.7)
III C	66 (22.9)
IV A	21 (7.3)
IV B	11 (3.8)
Tumor size (cm)	
≤ 5	105 (36.5)
5 < size ≤ 10	125 (43.4)
> 10	58 (20.1)
Child-Pugh score	
A	260 (90.3)
B	28 (9.7)
AFP (ng/mL)	
≤ 200	161 (57.7)
> 200	118 (42.3)

HBV: Hepatitis B virus; TNM: Tumor-node-metastasis; AFP: Alpha-fetoprotein.

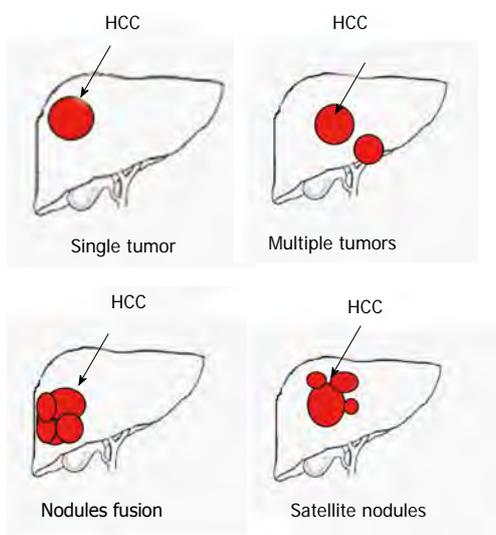


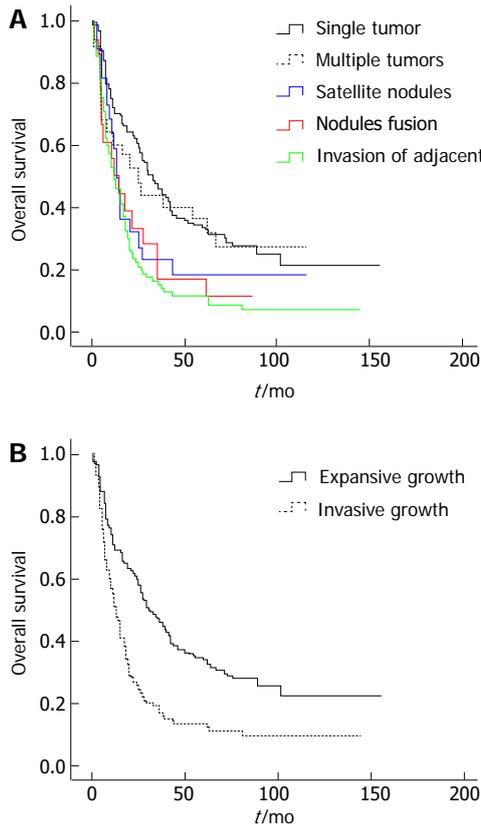
Figure 1 Growth characteristics of hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

number and invasive growth characteristics as follows: single tumor, multiple tumors, satellite nodules (including perforation the tumor encapsulation), nodule fusion (including diffuse growth lack tumor encapsulation) and invasion of adjacent organs (tumor with direct invasion of adjacent organs other than the gallbladder or with perforation of visceral peritoneum) (Figure 1). No significant difference in survival was observed between patients with a single tumor and patients with multiple tumors (Figure 2A). Moreover, no significant difference in survival was

**Table 2 Analysis of factors affecting survival**

	Death/all cases	Median survival time (95%CI)	<i>P</i> value
Age (yr)			0.612
≤ 60	165/208	19 (13.470-24.530)	
> 60	63/80	23 (15.988-30.012)	
Gender			0.605
Male	36/49	20 (6.283-33.717)	
Female	193/239	20 (15.131-24.869)	
Tumor size (cm)			< 0.001
≤ 5	67/105	34 (17.568-50.432)	
5 < size ≤ 10	107/125	17 (8.965-25.035)	
> 10	55/58	8 (4.890-11.110)	
Tumor status			< 0.001
T1	90/126	33 (24.933-41.067)	
T2	14/22	25 (0.000-73.264)	
T3a	26/31	15 (5.184-24.816)	
T3b	24/27	8 (5.470-10.530)	
T4	75/82	12 (7.905-16.095)	
Regional lymph node metastasis			< 0.001
No	205/264	22 (16.882-27.118)	
Yes	24/24	8 (3.999-12.001)	
Distant metastasis			< 0.001
No	217/276	22 (17.691-26.309)	
Yes	12/12	4 (2.303-5.697)	
Child-Pugh score			< 0.001
A	203/260	23 (18.485-27.515)	
B	26/28	7 (2.851-11.149)	
AFP (ng/mL)			< 0.001
≤ 200	118/161	28 (21.162-34.838)	
> 200	102/118	12 (8.198-15.802)	
Expansive growth and invasive growth			< 0.001
Single tumor	103/143	33 (24.408-41.592)	
Multiple tumors	18/25	25 (8.680-41.320)	
Satellite nodules (including perforation tumor encapsulation)	18/22	13 (10.242-15.758)	
Nodules fusion (including diffuse growth which lack tumor encapsulation)	16/18	12 (3.684-20.316)	
Direct invasion of adjacent organs other than the gallbladder or with perforation of visceral peritoneum	74/80	12 (7.956-16.044)	
Disseminative growth			< 0.001
Without vascular or regional lymph node involvement	155/210	28 (21.928-34.072)	
Vascular involvement	42/46	8 (4.979-11.021)	
Regional lymph node involvement	21/21	10 (4.019-15.981)	
Distant metastasis	12/12	4 (2.303-5.697)	
EID stage			< 0.001
I	96/140	36 (27.626-44.374)	
II	84/99	16 (11.450-20.550)	
III	38/38	8 (4.375-11.625)	
IV	11/11	5 (3.436-6.564)	
TNM stage			< 0.001
I	84/119	35 (26.448-43.552)	
II	14/22	25 (0.000-73.264)	
III A	19/24	16 (2.557-29.443)	
III B	22/25	8 (1.880-14.120)	
III C	58/66	15 (10.033-19.967)	
IV A	21/21	10 (4.019-15.981)	
IV B	11/11	5 (3.436-6.564)	

AFP: Alpha-fetoprotein; EID: Expansive-Invasive-Disseminative growth; TNM: Tumor-node-metastasis.

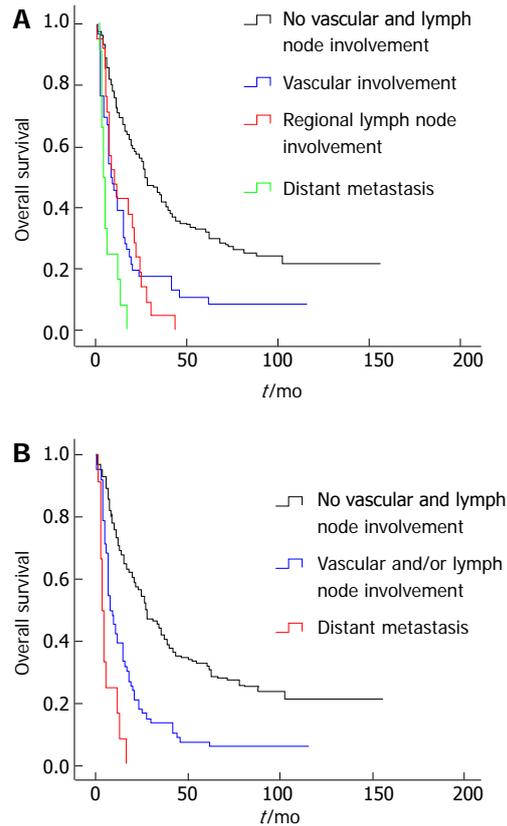


**Figure 2** Overall survival curves for patients with expansive tumor growth and invasive tumor growth. A: No significant difference in survival was observed between patients with a single tumor and patients with multiple tumors. Moreover, no significant difference in survival was observed among patients with satellite nodules, patients with nodule fusion and patients with tumor invasion of adjacent organs; B: Based on the data, patients were divided to two groups: expansive tumor growth (single tumor and multiple tumors) and invasive tumor growth (satellite nodules, nodule fusion and tumors with direct invasion of adjacent organs). The overall survival was  $57.398 \pm 4.873$  mo for patients with expansive tumor growth, while it was  $27.796 \pm 3.730$  mo for patients with invasive tumor growth ( $P < 0.001$ ).

observed among patients with satellite nodules, patients with nodule fusion or patients with tumor invasion of adjacent organs (Figure 2A). Based on the data, patients were divided into two groups: expansive tumor growth (single tumor and multiple tumors) and invasive tumor growth (satellite nodules, nodules fusion, and tumor with direct invasion of adjacent organs). The OS of patients with invasive tumor growth was shorter than that of patients with expansive tumor growth ( $P < 0.001$ , Figure 2B). The OS was  $57.398 \pm 4.873$  mo for patients with expansive tumor growth, while it was  $27.796 \pm 3.730$  mo for patients with invasive tumor growth. The median survival time of patients with expansive tumor growth and patients with invasive tumor growth were 30 and 13 mo, respectively.

**Survival analysis of disseminative growth**

Based on the extrahepatic metastatic tendency of tumor, the patients were divided to four groups: tumor without vascular and regional lymph node involvement; tumor

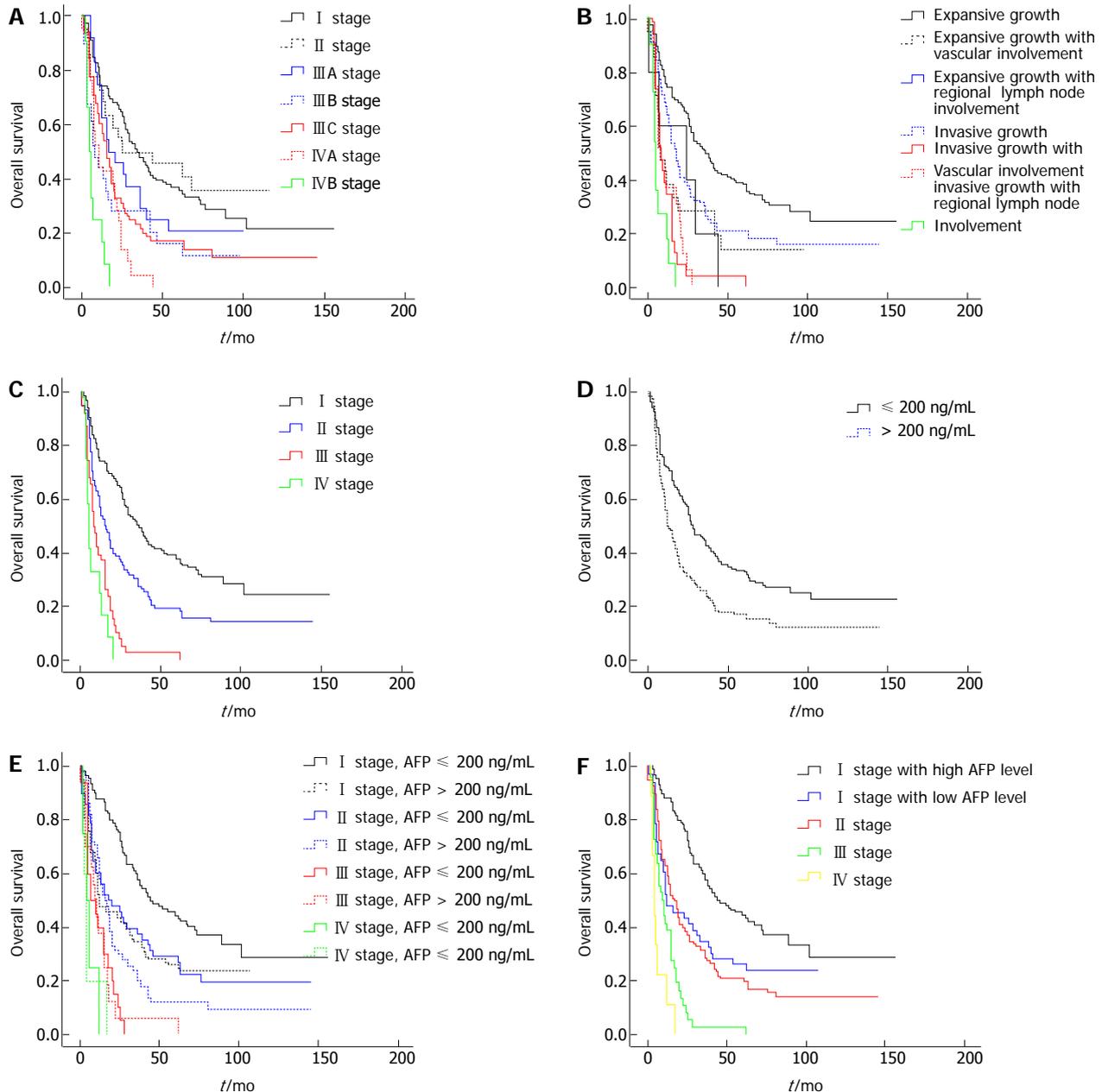


**Figure 3** Overall survival curves for patients with disseminative growth. A: No significant difference in survival was observed between patients with vascular involvement of tumor and patients with regional lymph node metastasis. The overall survival (OS) of patients with tumors that lacked vascular and regional lymph node involvement was longer than those of other groups, while the OS of patients with distant metastasis was shorter than those of other groups; B: Based on these data, patients were divided into three groups: tumors without vascular and regional lymph node involvement, tumors with vascular and/or regional lymph node involvement and distant metastasis.

with vascular involvement; regional lymph node metastasis; and distant metastasis. No significant differences in survival were observed between patients with vascular involvement of the tumor and patients with regional lymph node metastasis (Figure 3A). The OS of patients who lacked vascular and regional lymph node involvement was better than other groups, while the OS of patients with distant metastasis was shorter than those of other groups (Figure 3A). The OS of patients who lacked vascular and regional lymph node involvement was  $55.532 \pm 4.237$  mo, while the OS of patients with distant metastasis was  $6.417 \pm 1.395$  mo. The OS of patients with vascular and regional lymph node tumor involvement was  $21.667 \pm 4.773$  and  $14.619 \pm 2.456$  mo, respectively. The median survival times for each group were 28, 8, 10 and 4 mo, respectively. Based on these data, the patients were divided into three groups: tumor without vascular and regional lymph node involvement; tumor with vascular and/or regional lymph node involvement; and distant metastasis (Figure 3B).

**New classification of HCC**

According to the 7<sup>th</sup> edition TNM classification, no sig-



**Figure 4** New classification for hepatocellular carcinoma. A: Overall survival (OS) curves for patients based on tumor-node-metastasis system classification; B: OS curves for patients with expansive growth, invasive growth and disseminative growth; C: OS curves for patients based on Expansive-Invasive-Disseminative growth (EID) staging classification; D: OS curves for patients with different levels of alpha-fetoprotein (AFP); E: OS curves for patients based on EID staging classification and AFP; F: Overall survival curves for patients combined EID staging classification and AFP.

nificant difference in survival was observed between the groups with stage I or II HCC (Figure 4A). The OS of patients with stage I was  $59.460 \pm 5.806$  and  $55.585 \pm 10.289$  mo for patients with stage II. Moreover, no significant differences in survival were observed among the different stage III groups (Figure 4A). The OS of patients with stage IIIA, IIIB and IIIC were  $35.917 \pm 7.138$ ,  $24.760 \pm 6.250$  and  $31.996 \pm 5.457$  mo, respectively.

Patients were divided to seven groups based on the growth characteristics of the tumor: expansive growth, expansive growth with vascular involvement, expansive growth with regional lymph node involvement, invasive growth, invasive growth with vascular involvement, inva-

sive growth with regional lymph node involvement, and distant metastasis. The OS of each group was  $62.632 \pm 5.415$ ,  $25.762 \pm 7.024$ ,  $21.200 \pm 7.794$ ,  $39.533 \pm 5.840$ ,  $11.478 \pm 2.635$ ,  $12.653 \pm 2.076$  and  $6.727 \pm 1.490$  mo, respectively. There were significant differences in survival among patients with expansive tumor growth, invasive tumor growth and disseminative tumor growth. No significant difference in survival was observed between patients with expansive tumor growth along with vascular and/or regional lymph node involvement and patients with invasive tumor growth who lacked vascular and regional lymph node involvement (Figure 4B).

These data enable the proposal of a new staging sys-

**Table 3** Expansive-Invasive-Disseminative growth staging classification

Phase	Growth characteristics
I	Expansive tumor growth (single tumor and multiple tumors)
II	Expansive tumor growth along with vascular and/or regional lymph node involvement Invasive tumor growth (satellite nodules, nodule fusion and tumor direct invasion of adjacent organs)
III	Invasive tumor growth along with vascular/regional lymph node involvement
IV	Distant metastasis of tumor

tem: the Expansive-Invasive-Disseminative growth (EID) staging classification, which comprises four stages that select the best candidates for the best therapies currently available. Stage I includes patients with expansive tumor growth. Stage II has two subgroups: the first group consists of patients with expansive tumor growth along with vascular and/or regional lymph node involvement, and the second group includes patients with invasive tumor growth. Stage III includes patients with invasive tumor growth along with vascular/regional lymph node involvement. Stage IV includes patients with distant metastasis (Figure 4C). The OS values of patients at each stage were  $62.637 \pm 5.453$ ,  $36.880 \pm 7.779$ ,  $12.053 \pm 1.796$  and  $6.727 \pm 1.490$  mo, respectively. The overall median survival times of patients at each stage were 36, 16, 8 and 5 mo, respectively.

Table 2 showed the univariate analysis of the factors measured association with survival. The tumor characteristics that were statistically significant were tumor size, AFP, Child Pugh score, growth pattern, TNM stage and EID stage. Multivariate analyses identified EID stage and AFP as independent factors associated with OS ( $P < 0.001$ ,  $P = 0.008$ , respectively). There was no significant difference between the TNM stage and Child Pugh score. The OS of patients with high levels of AFP (AFP > 200 ng/mL;  $56.229 \pm 4.849$  mo) was shorter than that of patients with low levels of AFP ( $33.208 \pm 4.212$  mo;  $P < 0.001$ , Figure 4D). Moreover, the OS of stage I patients with high levels of AFP was shorter than that of patients with low levels of AFP ( $72.240 \pm 6.793$  mo,  $37.804 \pm 6.054$  mo, respectively,  $P < 0.001$ ). No significant difference in survival was observed between I stage patients with high levels of AFP and II stage patients. Dramatically, for stage II, III and IV patients, no significant difference in survival was observed between patients with high levels of AFP and patients with low levels of AFP (Figure 4E). Thus, AFP levels only affect the OS of stage I patients (Figure 4F).

## DISCUSSION

There are four main factors affecting the prognosis of HCC: (1) the stage, aggressiveness and growth rate of the tumor; (2) the general health of the patient; (3) the liver function of the patient; and (4) the specific intervention<sup>[20]</sup>. A number of staging systems have been devised for patients with HCC. Each staging system includes variables which evaluate one or more of the first 3 factors listed above. For example, the TNM staging system eval-

uates only the tumor characteristics, whereas the Child Pugh score provides information regarding liver function. Some of the characteristics of HCC tumors include the tumor size, tumor number, aggressiveness of growth, vascular involvement, regional lymph node metastasis, and extrahepatic spread. Although the TNM and BCLC staging classification for HCC were proposed based on certain tumor characteristics, no staging system systematically evaluates the effect of tumor growth patterns on the clinical outcome of patients with HCC.

In this study, 288 postoperative patients with HCC were studied, and the tumor growth patterns were divided into three types: (1) expansive growth (single tumor and multiple tumors without invasive and disseminative growth); (2) invasive growth (satellite nodules including perforation of the tumor encapsulation, nodule fusion including diffuse growth that lacks tumor encapsulation, and tumors with direct invasion of adjacent organs); and (3) disseminative growth (vascular involvement, regional lymph node metastasis and distant metastasis). Cheng *et al*<sup>[6]</sup> reported that the lack of tumor encapsulation was an independent factor for HCC. Other research showed that the presence of satellite nodules was an independent factor for the long-term survival of patients with HCC after curative resection<sup>[9,21]</sup>. The OS of patients with invasive tumor growth was shorter than that of patients with expansive tumor growth. No significant difference in survival was observed between patients with vascular involvement and patients with regional lymph node metastasis. The OS of patients with expansive tumor growth was longer than those of other groups, while the OS of patients with distant metastasis was shorter than those of other groups. No significant differences in survival were observed between patients with expansive tumor growth with vascular and/or regional lymph node involvement and patients with invasive tumor growth that lacked vascular and regional lymph node involvement.

These data enable the proposal of a new staging system to select the best candidates for the best therapies currently available: the four-stage EID staging classification (Table 3). Stage I includes patients with expansive tumor growths. Stage II has two subgroups: the first group contains patients with expansive tumor growth along with vascular and/or regional lymph node involvement, and the second group consists of patients with invasive tumor growth. Stage III includes patients with invasive tumor growth along with vascular/regional lymph node involvement. Stage IV comprises patients with distant tumor metastasis. The OS values of each stage

**Table 4** Characteristics of patients as defined by Expansive-Invasive-Disseminative growth stage *n* (%)

Characteristics	I	II	III	IV
Age (yr)				
≤ 60	96 (68.6)	78 (78.8)	26 (68.4)	8 (72.9)
> 60	44 (31.4)	21 (21.2)	12 (31.6)	3 (27.3)
Gender				
Female	27 (19.3)	15 (15.2)	5 (13.2)	2 (18.2)
Male	113 (80.7)	84 (84.8)	33 (86.8)	9 (81.8)
HBV infection	105 (75.0)	75 (75.8)	23 (60.5)	8 (72.7)
TNM stage				
I	117 (83.6)	2 (2.0)	0	0
II	12 (8.6)	10 (10.1)	0	0
III A	9 (6.4)	15 (15.2)	0	0
III B	0	20 (20.2)	5 (13.2)	0
III C	2 (1.4)	46 (44.5)	18 (47.4)	0
IV A	0	6 (6.1)	15 (39.5)	0
IV B	0	0	0	11 (100)
Tumor size (cm)				
≤ 5	71 (50.7)	27 (27.3)	5 (13.2)	2 (18.2)
5 < size ≤ 10	52 (37.1)	50 (50.5)	18 (47.4)	5 (45.5)
> 10	17 (12.1)	22 (22.2)	15 (39.5)	4 (36.4)
Child-Pugh score				
A	128 (91.4)	92 (92.9)	30 (78.9)	10 (90.9)
B	12 (8.6)	7 (7.1)	8 (21.1)	1 (9.1)
AFP (ng/mL)				
≤ 200	91 (66.4)	46 (47.4)	20 (55.6)	4 (44.4)
> 200	46 (33.6)	51 (52.6)	16 (44.4)	5 (55.6)

HBV: Hepatitis B virus; TNM: Tumor-node-metastasis; AFP: Alpha-fetoprotein.

were  $62.637 \pm 5.453$ ,  $36.880 \pm 7.779$ ,  $12.053 \pm 1.796$  and  $6.727 \pm 1.490$  mo, respectively. The median survival times of patients at each stage were 36, 16, 8 and 5 mo, respectively.

Univariate analysis showed that the statistically significant factors were EID staging classification and AFP (Table 4). There were no statistically significant correlative differences between OS and the Child-Pugh score. There is a substantial amount of research showing that the Child-Pugh score and AFP values > 200 ng/mL are independent factors for HCC<sup>[6-8,15,22]</sup>. In fact, patients with a C Child-Pugh score were excluded for this study to eliminate the effects of poor liver function on long-term outcome. The OS of stage I patients with high levels of AFP was shorter than that of patients with low levels of AFP. There is research showing that the Japan Integrated Staging Score and BCLC staging system combined with AFP levels may serve as a better staging system for early-stage HCC patients<sup>[22,23]</sup>. Dramatically, for patients in stages II, III and IV, no significant difference in survival was observed between patients with high levels of AFP and patients with low levels of AFP. Thus, AFP levels likely only affect the OS of stage I patients.

In conclusion, the EID staging classification is a simple and efficacious prognostic model for postoperative patients with HCC. Because the EID staging classification is easily obtained and objective, we propose it for widespread use in clinical practice as a staging system for postoperative patients with HCC.

## COMMENTS

### Background

The tumor-node-metastasis (TNM) system fails to include comprehensive characteristics of the tumor, especially the tumor's growth pattern. Thus, it is crucial to design a system to evaluate the effects of tumor characteristics on the clinical outcome of resectable patients with hepatocellular carcinoma (HCC).

### Research frontiers

The characteristics of HCC tumors include the tumor size, tumor number, aggressiveness of growth, vascular involvement, regional lymph node metastasis, and extrahepatic spread. Although the TNM and Barcelona Clinic Liver Cancer staging classification for HCC were proposed based on certain tumor characteristics, no staging system systematically evaluates the effect of tumor growth patterns on the clinical outcome of patients with HCC.

### Innovations and breakthroughs

These data enable the proposal of a new staging system to select the best candidates for the best therapies currently available: the four-stage Expansive-Invasive-Disseminative growth (EID) staging classification. Stage I includes patients with expansive tumor growths. Stage II has two subgroups: the first group contains patients with expansive tumor growth along with vascular and/or regional lymph node involvement, and the second group consists of patients with invasive tumor growth. Stage III includes patients with invasive tumor growth along with vascular/regional lymph node involvement.

### Applications

The EID staging classification is a simple and efficacious prognostic model for postoperative patients with HCC. Because the EID staging classification is obtained easily, authors propose it for postoperative patients with HCC.

### Terminology

Expansive growth includes single tumor and multiple tumors without invasive and disseminative growth; invasive growth consists of satellite nodules including perforation of the tumor encapsulation, nodule fusion including diffuse growth that lacks tumor encapsulation and tumors with direct invasion of adjacent organs; disseminative growth contains vascular involvement, regional lymph node metastasis and distant metastasis

### Peer review

The prognostic significance of the new staging method was confirmed by the detailed statistical analysis of 288 patients treated in a single facility, being compared with other staging systems previously proposed. This clinical study is interesting and novel. The author found a simply and efficacy prognostic model for postoperative patients with HCC.

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## Impact of intraoperative blood loss on survival after curative resection for gastric cancer

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### Abstract

**AIM:** To elucidate the potential impact of intraoperative blood loss (IBL) on long-term survival of gastric cancer patients after curative surgery.

**METHODS:** A total of 845 stage I-III gastric cancer patients who underwent curative gastrectomy between January 2003 and December 2007 in our center were enrolled in this study. Patients were divided into 3 groups according to the amount of IBL: group 1 (< 200 mL), group 2 (200-400 mL) and group 3 (> 400 mL). Clinicopathological features were compared among the three groups and potential prognostic factors were analyzed. The Log-rank test was used to assess statistical differences between the groups. Independent prognostic factors were identified by the Cox proportional haz-

ards regression model. Stratified analysis was used to investigate the impact of IBL on survival in each stage. Cancer-specific survival was also compared among the three groups by excluding deaths due to reasons other than gastric cancer. Finally, we explored the possible factors associated with IBL and identified the independent risk factors for IBL  $\geq$  200 mL.

**RESULTS:** Overall survival was significantly influenced by the amount of IBL. The 5-year overall survival rates were 51.2%, 39.4% and 23.4% for IBL less than 200 mL, 200 to 400 mL and more than 400 mL, respectively (< 200 mL vs 200-400 mL,  $P < 0.001$ ; 200-400 mL vs > 400 mL,  $P = 0.003$ ). Age, tumor size, Borrmann type, extranodal metastasis, tumour-node-metastasis (TNM) stage, chemotherapy, extent of lymphadenectomy, IBL and postoperative complications were found to be independent prognostic factors in multivariable analysis. Following stratified analysis, patients staged TNM I-II and those with IBL less than 200 mL tended to have better survival than those with IBL not less than 200 mL, while patients staged TNM III, whose IBL was less than 400 mL had better survival. Tumor location, tumor size, TNM stage, type of gastrectomy, combined organ resection, extent of lymphadenectomy and year of surgery were found to be factors associated with the amount of IBL, while tumor location, type of gastrectomy, combined organ resection and year of surgery were independently associated with IBL  $\geq$  200 mL.

**CONCLUSION:** IBL is an independent prognostic factor for gastric cancer after curative resection. Reducing IBL can improve the long-term outcome of gastric cancer patients following curative gastrectomy.

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**Key words:** Gastric carcinoma; Intraoperative blood loss; Blood transfusion; Postoperative complication; Prognosis

**Core tip:** Intraoperative blood loss (IBL) has been shown to be associated with poor outcome in various types of malignancy. In this study, we found that the overall survival of gastric cancer patients was significantly affected by the amount of IBL, and IBL was an independent prognostic factor in multivariate analysis. We suggest that meticulous surgery and new surgical methods such as the application of an ultrasonic scalpel in lymph node dissection should be used to decrease the amount of IBL and improve the long-term outcome of gastric cancer patients following curative gastrectomy.

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## INTRODUCTION

Radical gastrectomy with regional lymph node dissection is the only possible curative treatment for gastric cancer<sup>[1]</sup>. Even after R0 resection, a significant number of patients suffer from recurrence, especially those with advanced gastric cancer<sup>[2-4]</sup>. Tumor depth and lymph node status are well-known prognostic factors, and patient age and performance status have also been reported to have an impact on the long-term outcome of patients<sup>[5-7]</sup>. Besides these factors, a number of potential prognostic factors have been reported in recent years, such as perioperative blood transfusion and intraoperative blood loss (IBL)<sup>[8-11]</sup>.

The impact of IBL on long-term outcome has previously been reported in patients with colorectal cancer, prostate cancer and pancreas cancer<sup>[12-14]</sup>. However, there are few reports assessing the relationship between IBL and long-term outcome in gastric cancer patients. Dhar *et al*<sup>[10]</sup> reported that more than 500 mL blood loss during surgery was an independent predictor of survival in gastric cancer patients with transmural depth invasion. Kamei *et al*<sup>[11]</sup> demonstrated that IBL was a crucial risk factor for peritoneal recurrence after curative resection for advanced gastric cancer. Unfortunately, the numbers of patients included in these aforementioned studies were small, and no further meticulous analysis was performed to explore the correlation between the prognosis of gastric cancer patients and the accurate amount of IBL.

The aim of the present study is to elucidate the potential impact of IBL on the long-term survival of gastric cancer patients after curative surgery in a single high-volume center in China.

## MATERIALS AND METHODS

### Patients

The surgical and pathological data of 845 patients with

gastric cancer who had undergone curative gastrectomy (R0 resection) with lymph node dissection and had been followed up between January 2003 and December 2007 at Tianjin Medical University Cancer Institute and Hospital were reviewed in this study. All the patients had been histologically diagnosed with adenocarcinoma of the stomach. Patients who previously underwent gastric surgery or received preoperative chemotherapy were excluded. Patients with distant metastasis were also excluded. The study population consisted of 845 patients, 607 males (71.8%) and 238 females (28.2%) with a median age of 62 years (range, 23-89 years).

### Surgical treatment and perioperative management

All the patients underwent gastrectomy with D1 or D2 lymph node dissection. The choice of surgical procedure for reconstruction was made by the surgeon. Resection margin was pathologically confirmed as negative. Postoperative adjuvant chemotherapy was administered according to tumor stage, physical condition and the patient's willingness. Chemotherapeutics consisted of 5-fluorouracil, leucovorin and oxaliplatin. Radiotherapy was not administered in the present study.

IBL was visually estimated according to the weight or volume of blood absorbed by gauze and suction pump by anesthesiologists immediately after surgery. We obtained this information from anesthesia records. IBL ranged from 50 to 1500 mL and the median IBL was 200 mL for the whole group. The patients were divided into 3 groups according to the amount of IBL: group 1 (< 200 mL), group 2 (200-400 mL) and group 3 (> 400 mL). The entire transfusion history during hospital stay for surgery was recorded. Patients whose perioperative hemoglobin was less than 70 g/L or who lost a lot of blood during surgery were routinely given a red blood cell transfusion. Of the 845 patients, 211 had a perioperative red blood cell transfusion, and the remaining 634 did not receive a transfusion. Postoperative complications during hospitalization only included these directly associated with surgery, such as hemorrhage, wound dehiscence, anastomotic leak, pancreatic fistula, lymphatic fistula and abdominal or wound infection.

### Evaluation of clinicopathological variables and survival

The clinicopathological features studied included gender, age, tumor location, tumor size, Borrmann type, histology, extranodal metastasis (EM), type of gastrectomy, combined organ resection, postoperative chemotherapy, tumour-node-metastasis (TNM) stage, extent of lymphadenectomy, postoperative complications, perioperative transfusion, and IBL. Clinicopathological features were first compared among the three groups and the impact of each factor on survival was evaluated to identify independent prognostic factors. We next determined whether IBL influenced cancer-specific survival by comparing overall survival among the three groups by excluding deaths due to reasons other than gastric cancer. Finally, we explored the possible factors associated with IBL and identified

**Table 1** Case characteristics *n* (%)

Characteristics	IBL (mL)			$\chi^2$	P value
	< 200	200-400	> 400		
IBL (mean $\pm$ SD)	99.3 $\pm$ 25.0	223.2 $\pm$ 41.6	484.4 $\pm$ 179.9		
Gender				4.307	0.116
Male	269 (70.2)	285 (71.6)	53 (71.8)		
Female	114 (29.8)	113 (28.4)	11 (28.2)		
Age (yr)				2.488	0.288
$\leq$ 65	230 (60.1)	227 (57.0)	32 (50.0)		
> 65	153 (39.9)	171 (43.0)	32 (50.0)		
Tumor location				40.555	< 0.001
Lower 1/3	205 (53.5)	148 (37.2)	14 (21.9)		
Middle 1/3	36 (9.4)	41 (10.3)	6 (9.4)		
Upper 1/3	98 (25.6)	164 (41.2)	34 (53.1)		
2/3 or more	44 (11.5)	45 (11.3)	10 (15.6)		
Tumor size				17.677	< 0.001
< 5 cm	180 (47.0)	155 (38.9)	13 (20.3)		
$\geq$ 5 cm	203 (53.0)	243 (61.1)	51 (79.7)		
Borrmann type				5.180	0.075
I / II	169 (44.1)	153 (38.4)	33 (51.6)		
III / IV	214 (55.9)	245 (61.6)	31 (48.4)		
Histology				0.982	0.612
Differentiated	121 (31.6)	139 (34.9)	21 (32.8)		
Undifferentiated	262 (68.4)	259 (65.1)	43 (67.2)		
Extranodal metastasis				1.963	0.375
Positive	59 (15.4)	71 (17.8)	14 (21.9)		
Negative	324 (84.6)	327 (82.2)	50 (78.1)		
Depth of invasion				14.719	0.023
pT1	14 (3.7)	11 (2.8)	0 (0.0)		
pT2	53 (13.8)	44 (11.1)	0 (0.0)		
pT3	21 (5.5)	28 (7.0)	6 (9.4)		
pT4	295 (77.0)	315 (79.1)	58 (90.6)		
Lymph node metastasis				15.793	0.015
pN0	173 (45.2)	146 (36.7)	19 (29.7)		
pN1	56 (14.6)	82 (20.6)	9 (14.1)		
pN2	85 (22.2)	87 (21.9)	15 (23.4)		
pN3	69 (18.0)	83 (20.9)	21 (32.8)		
TNM stage				15.313	0.004
I	53 (13.8)	43 (10.8)	0 (0.0)		
II	132 (34.5)	118 (29.6)	19 (29.7)		
III	198 (51.7)	237 (59.5)	45 (70.3)		
Chemotherapy				2.036	0.361
Yes	104 (27.2)	119 (29.9)	14 (21.9)		
No	279 (72.8)	279 (70.1)	50 (78.1)		
Type of gastrectomy				37.357	< 0.001
Total	51 (13.3)	117 (29.4)	24 (37.5)		
Subtotal	332 (86.7)	281 (70.6)	40 (62.5)		
Combined organ resection				22.256	< 0.001
Yes	16 (4.2)	38 (9.5)	13 (20.3)		
No	367 (95.8)	360 (90.5)	51 (79.7)		
Extent of lymphadenectomy				7.230	0.027
D2 and D2+	189 (49.3)	188 (47.2)	20 (31.3)		
D1	194 (50.7)	210 (52.8)	44 (68.8)		
Postoperative complications				7.500	0.024
Present	20 (5.2)	34 (8.5)	9 (14.1)		
Absent	363 (94.8)	364 (91.5)	55 (85.9)		

IBL: Intraoperative blood loss; TNM: Tumour-node-metastasis.

the independent risk factors for IBL  $\geq$  200 mL. The tumors were staged according to the 7<sup>th</sup> edition Union for International Cancer Control TNM classification system, whereas lymphadenectomy and lymph node stations were defined according to the 3<sup>rd</sup> English Edition of the Japanese Classification of Gastric Carcinoma. Tumors were

classified into two groups based on histology: differentiated type including papillary, well or moderately differentiated adenocarcinoma; and undifferentiated type including poorly differentiated or undifferentiated adenocarcinoma, signet ring cell carcinoma and mucinous carcinoma.

### Follow-up

The patients were followed up every 3 mo up to 2 years after surgery, then every 6 mo up to 5 years, and then every year or until death. Physical examination, laboratory tests, imaging and endoscopy were performed at each visit. The median follow-up was 39 mo (range 1-103 mo), and the last follow-up date was December 20, 2012. The overall survival rate was calculated from the day of surgical resection until time of death or final follow-up.

### Statistical analysis

Categorical variables were analyzed by means of the  $\chi^2$  or Fisher's exact test. Overall survival curves were calculated using the Kaplan-Meier method based on the length of time between primary surgical treatment and final follow-up or death; the Log-rank test was used to assess statistical differences between the groups. Independent prognostic factors were identified by the Cox proportional hazards regression model. One-way analysis of variance (ANOVA) analysis or *t* test was used in univariate analysis to identify possible factors associated with IBL. Independent risk factors for IBL  $\geq$  200 mL were determined by logistic regression. *P* < 0.05 was considered statistically significant. The statistical analysis was performed using the statistical program SPSS 17.0 (SPSS, Chicago, IL, United States).

## RESULTS

### Clinicopathological features

Of the 845 patients, 397 (47.0%) patients underwent D2 or greater lymph node dissection, and the remaining 448 (53.0%) patients underwent D1 lymph node dissection. Sixty-seven patients underwent gastrectomy combined with other organ resections and 237 patients received postoperative adjuvant chemotherapy.

The patients were divided into three groups according to IBL (Table 1). The mean IBL was 99.4 mL in group 1, 223.2 mL in group 2 and 484.4 mL in group 3. There were no statistical differences in gender, age, Borrmann type, histology, EM and postoperative chemotherapy among the three groups. Tumors located in the upper one-third were more frequent in group 2 and group 3, while in group 1, 53.5% of tumors were located in the lower one-third. The incidence of postoperative complications and the ratios of tumors with a diameter  $\geq$  5 cm increased when the amount of IBL was high. Total gastrectomy and combined organ resection were more frequently performed in group 3 than in group 1 and group 2. Patients in group 2 and group 3 were more likely to have advanced tumor (T), node (N), and TNM stage than

Table 2 Survival analysis of all patients with gastric cancer

Characteristics	n (%)	5-yr OS	Univariate analysis		Multivariate analysis	
			$\chi^2$	P value	HR (95%CI)	P value
Gender			1.609	0.205		
Male	607 (71.8)	42.20%				
Female	238 (28.2)	47.10%				
Age (yr)			21.037	< 0.001		
≤ 65	489 (57.9)	50.10%			1 (ref)	
> 65	356 (42.1)	34.60%			1.372 (1.140-1.652)	0.001
Tumor location			26.417	< 0.001		
Lower 1/3	367 (43.4)	50.10%			1 (ref)	
Middle 1/3	83 (9.8)	45.80%			0.978 (0.680-1.407)	0.905
Upper 1/3	296 (35.0)	39.50%			0.931 (0.741-1.169)	0.538
2/3 or more	99 (11.7)	29.30%			1.149 (0.832-1.586)	0.398
Tumor size			58.693	< 0.001		
< 5 cm	348 (41.2)	57.80%			1 (ref)	
≥ 5 cm	497 (58.8)	33.60%			1.411 (1.152-1.730)	0.001
Borrmann type			13.517	< 0.001		
I / II	355 (42.0)	50.40%			1 (ref)	
III/IV	490 (58.0)	38.60%			1.285 (1.062-1.556)	0.010
Histology			6.783	0.009		
Differentiated	281 (33.3)	49.80%			1 (ref)	
Undifferentiated	564 (66.7)	40.40%			1.151 (0.939-1.412)	0.176
Extranodal metastasis			52.773	< 0.001		
Negative	701 (83.0)	47.50%			1 (ref)	
Positive	144 (17.0)	24.30%			1.543 (1.236-1.925)	< 0.001
TNM stage			147.103	< 0.001		
I	96 (11.4)	82.30%			1 (ref)	
II	269 (31.8)	58.40%			2.253 (1.362-3.727)	0.002
III	480 (56.8)	27.50%			4.736 (2.898-7.740)	< 0.001
Chemotherapy			10.999	0.001		
Yes	237 (28.0)	50.60%			1 (ref)	
No	608 (72.0)	40.80%			1.357 (1.093-1.684)	0.006
Extent of lymphadenectomy			6.668	0.010		
D2 and D2+	397 (47.0)	48.40%			1 (ref)	
D1	448 (53.0)	39.30%			1.372 (1.126-1.671)	0.002
Type of gastrectomy			21.400	< 0.001		
Subtotal	653 (77.3)	47.00%			1 (ref)	
Total	192 (22.7)	31.80%			1.102 (0.849-1.430)	0.466
Combined organ resection			10.310	0.001		
No	778 (92.1)	44.60%			1 (ref)	
Yes	67 (7.9)	31.30%			1.116 (0.811-1.536)	0.501
Intraoperative blood loss			29.175	< 0.001		
< 200 mL	383 (45.3)	51.20%			1 (ref)	
200-400 mL	398 (47.1)	39.40%			1.242 (1.017-1.516)	0.033
> 400 mL	64 (7.6)	23.40%			1.590 (1.140-2.217)	0.006
Perioperative transfusion			6.145	0.013		
No	634 (75.0)	45.70%			1 (ref)	
Yes	211 (25.0)	37.00%			0.962 (0.748-1.180)	0.708
Postoperative complications			28.320	< 0.001		
Absent	782 (92.5)	44.90%			1 (ref)	
Present	63 (7.5)	27.00%			2.096 (1.525-2.881)	< 0.001

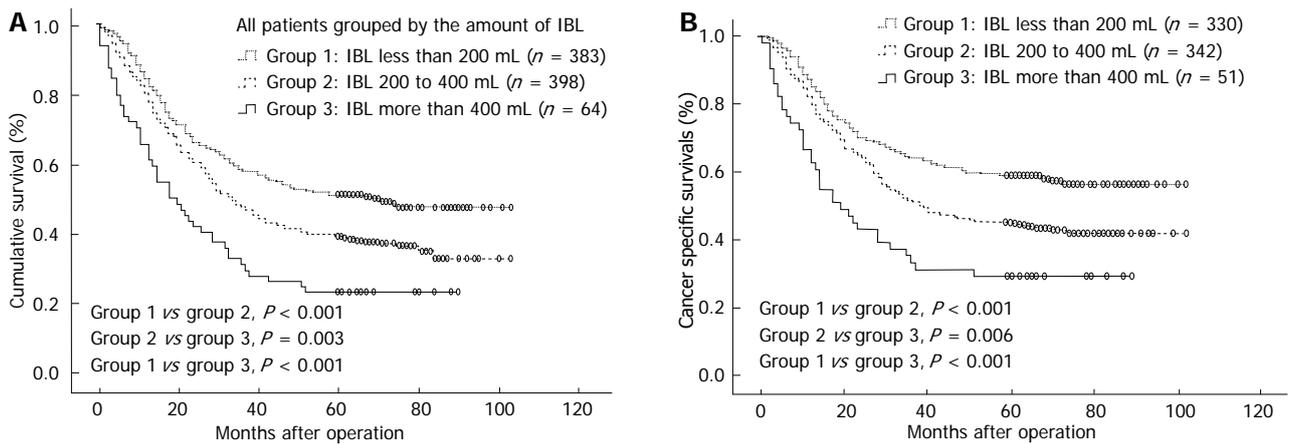
OS: Overall survival; TNM: Tumour-node-metastasis.

patients in group 1.

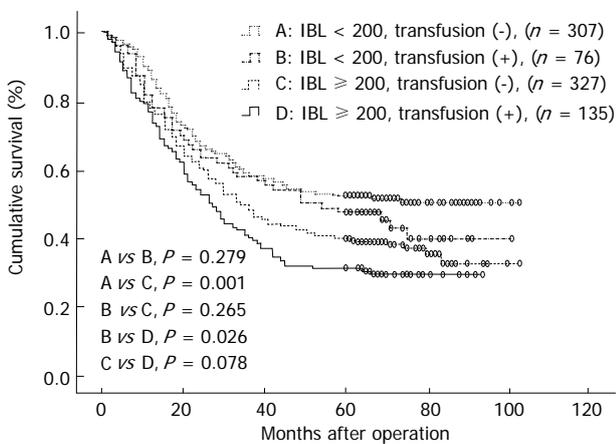
### Prognostic value of IBL in gastric cancer

Data from univariate and multivariate survival analyses are shown in Table 2. A total of 14 factors evaluated in the univariate analysis had a significant effect on survival: age (≤ 65 years *vs* > 65 years), tumor location, tumor size, Borrmann type (types I and II *vs* types III and IV), histology, EM, TNM stage, postoperative chemotherapy, type of gastrectomy, combined organ resection, extent of lymphadenectomy, IBL, perioperative transfusion and

postoperative complications. Gender did not influence survival. In multivariate analysis, age, tumor size, Borrmann type, EM, TNM stage, postoperative chemotherapy, extent of lymphadenectomy, postoperative complications and IBL were found to be independent prognostic factors for overall survival (OS). The 5-year OS rates were 51.2%, 39.4% and 23.4% for IBL < 200, 200-400, and > 400 mL, respectively, (< 200 mL *vs* 200-400 mL, *P* < 0.001; 200-400 mL *vs* > 400 mL, *P* = 0.001) (Figure 1A). When deaths due to factors other than gastric cancer were excluded, cancer-specific survival was still sig-



**Figure 1 Overall survival and cancer-specific curves for all patients grouped by intraoperative blood loss.** A: Overall survival curve; B: Cancer-specific survival curve. IBL: Intraoperative blood loss.



**Figure 2 Overall survival curves for all patients classified according to intraoperative blood loss and red blood cell transfusion.** IBL: Intraoperative blood loss.

nificantly influenced by IBL (Figure 1B). The 5-year OS rates for patients with red blood cell transfusion *vs* those without were 37.0% and 45.7% ( $P = 0.013$ ), respectively.

To assess the association between IBL and red blood cell transfusion, patients were categorized into 4 groups [IBL < 200 mL and transfusion (-); IBL < 200 mL, transfusion (+); IBL ≥ 200 mL, transfusion (-); IBL ≥ 200 mL, transfusion (+)], and OS was compared among these groups (Figure 2). As a blood loss of 200 mL was the median for the whole group, it was used for dichotomization in the statistical analysis. As a result, a IBL of 200 mL or more was a significant factor when excluding the influence of red blood cell transfusion ( $P = 0.001$ ;  $P = 0.026$ ). However, there was no significant difference in OS between patients with and without transfusion when the influence of IBL was excluded ( $P = 0.279$ ;  $P = 0.078$ ).

The results of the stratified analysis are shown in Table 3. In patients with TNM stage I, those with IBL less than 200 mL had significantly better survival than those with IBL 200-400 mL (Figure 3A). In the patients

**Table 3 Tumour-node-metastasis-stratified analysis of the overall survival**

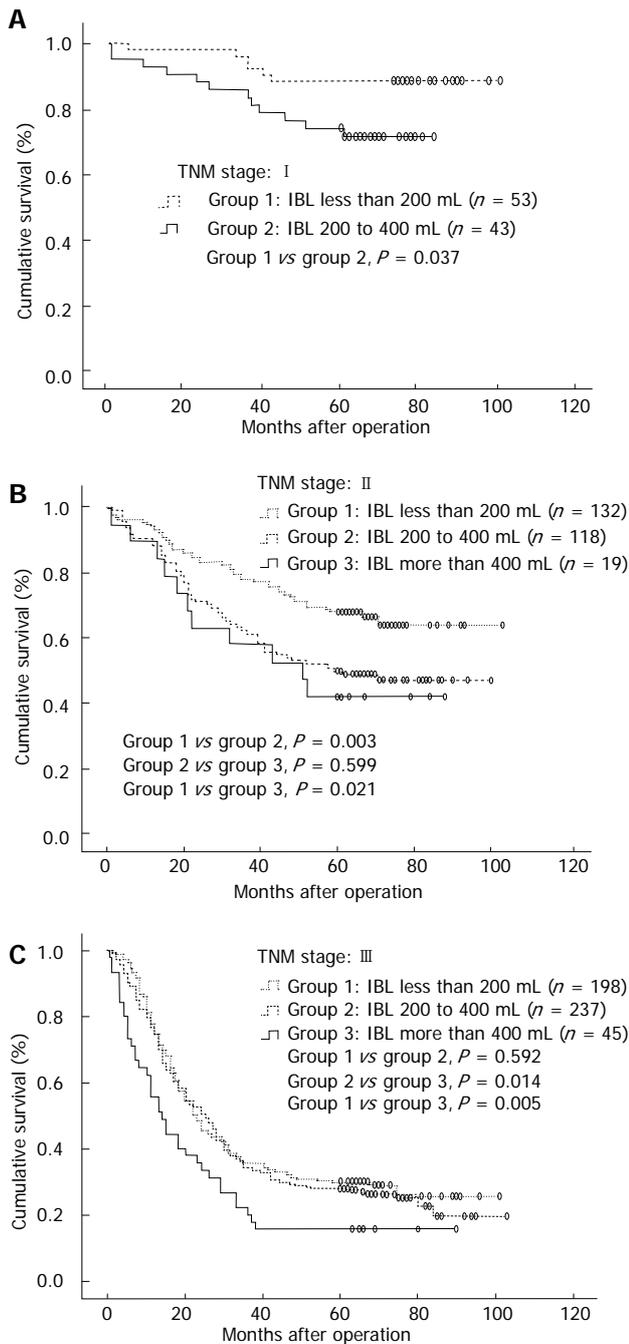
TNM	Group 1 <sup>1</sup>		Group 2 <sup>1</sup>		Group 3 <sup>1</sup>		$\chi^2$	P value
	n	5-yr OS	n	5-yr OS	n	5-yr OS		
I	53	88.7	43	74.4			4.538	0.037
II	132	68.2	118	50.0	19	42.1	10.763	0.005
III	198	29.8	237	27.8	45	15.6	8.035	0.018

<sup>1</sup>Group 1: IBL < 200 mL; Group 2: IBL 200-400 mL; Group 3: IBL > 400 mL. OS: Overall survival; TNM: Tumour-node-metastasis; IBL: Intraoperative blood loss.

staged with TNM II, those with IBL less than 200 mL had a significantly higher 5-year OS than those with IBL 200-400 mL or more than 400 mL, while there were no statistical differences in OS between those with IBL 200-400 mL and more than 400 mL (Figure 3B). For patients staged TNM III, OS did not differ significantly between those with IBL less than 200 mL and 200-400 mL, however, these patients had significantly higher 5-year OS than those with IBL more than 400 mL (Figure 3C).

**Risk factors associated with IBL**

Univariate analysis of factors associated with the amount of IBL is shown in Table 4. Following one-way ANOVA analysis or *t* test, tumor location, tumor size, TNM stage, type of gastrectomy, combined organ resection, extent of lymphadenectomy and year of surgery were found to be significant factors associated with the amount of IBL. Factors which had no influence on IBL were gender, age, Borrmann type, histology, and EM. As patients with IBL less than 200 mL had the best survival, we further identified the independent risk factors for IBL ≥ 200 mL. Factors significant in the univariate analysis were included in the multivariate analysis. Tumor location, type of gastrectomy, combined organ resection and year of surgery were found to be independent risk factors for IBL ≥ 200 mL in the multivariate analysis (Table 5).



**Figure 3 Overall survival curves.** A: 96 patients staged tumour-node-metastasis (TNM) I; B: 269 patients staged TNM II; C: 480 patients staged TNM III. IBL: Intraoperative blood loss.

## DISCUSSION

The prognosis of gastric cancer is mainly associated with tumor depth and lymph node status<sup>[15,6]</sup>. To improve the outcome of gastric cancer, standard surgery with D2 lymph node dissection is recommended<sup>[15,16]</sup>. However, even after curative gastrectomy with D2 dissection, the prognosis remains poor. In the present study, we evaluated the potential prognostic factors and found that IBL was significantly associated with the survival of patients

**Table 4 Association between clinicopathologic factors and the amount of intraoperative blood loss: univariate analysis**

Characteristics	n (%)	Amount of IBL (mL) (mean ± SD)	t/F	P value
Gender			1.770	0.077
Male	607 (71.8)	191.4 ± 128.6		
Female	238 (28.2)	175.2 ± 92.5		
Age (yr)			-1.128	0.260
≤ 65	489 (57.9)	182.9 ± 121.8		
> 65	356 (42.1)	192.3 ± 116.7		
Tumor location			12.455	< 0.001
Lower 1/3	367 (43.4)	160.9 ± 87.8		
Middle 1/3	83 (9.8)	179.5 ± 103.0		
Upper 1/3	296 (35.0)	213.2 ± 127.5		
2/3 or more	99 (11.7)	210.6 ± 177.5		
Tumor size			-4.129	< 0.001
< 5 cm	348 (41.2)	166.7 ± 92.8		
≥ 5 cm	497 (58.8)	200.9 ± 133.7		
Borrmann type			0.128	0.899
I / II	355 (42.0)	187.5 ± 127.0		
III / IV	490 (58.0)	186.4 ± 114.3		
Histology			-0.160	0.873
Differentiated	281 (33.3)	185.9 ± 107.7		
Undifferentiated	564 (66.7)	187.3 ± 125.3		
Extranodal metastasis			-1.040	0.299
Negative	701 (83.0)	184.9 ± 119.7		
Positive	144 (17.0)	196.3 ± 119.6		
TNM stage			4.974	0.007
I	96 (11.4)	154.2 ± 67.1		
II	269 (31.8)	183.3 ± 135.9		
III	480 (56.8)	195.4 ± 117.1		
Type of gastrectomy			-5.963	< 0.001
Subtotal	653 (77.3)	173.8 ± 102.3		
Total	192 (22.7)	231.2 ± 158.1		
Combined organ resection			-5.329	< 0.001
Absent	778 (92.1)	180.5 ± 110.9		
Present	67 (7.9)	260.4 ± 180.0		
Extent of lymphenectomy			-2.676	0.008
D2 and D2+	397 (47.0)	175.2 ± 95.4		
D1	448 (53.0)	197.2 ± 136.9		
Year of surgery			-2.494	0.013
2003-2005	489 (57.9)	195.1 ± 133.6		
2006-2007	356 (42.1)	174.3 ± 97.6		

TNM: Tumour, node, metastasis; IBL: Intraoperative blood loss.

**Table 5 Multivariate analysis of risk factors for intraoperative blood loss ≥ 200 mL**

Feature	HR	95%CI	P value
Tumor location Upper 1/3 and 2/3 or more vs lower and middle 1/3	1.717	1.272-2.317	< 0.001
Tumor size ≥ 5 cm vs < 5 cm	1.129	0.833-1.513	0.434
TNM stage III vs I, II	1.174	0.872-1.580	0.290
Extent of gastrectomy D1 vs D2 and D2+	1.161	0.860-1.566	0.330
Type of gastrectomy Total vs subtotal	2.501	1.707-3.663	< 0.001
Combined organ resection Present vs absent	1.996	1.089-3.659	0.025
Year of surgery 2003-2005 vs 2006-2007	1.452	1.080-1.954	0.014

TNM: Tumour, node, metastasis.

with gastric cancer after curative resection.

IBL has been reported to be associated with the prognosis of many malignant tumors<sup>[12-14]</sup>. Mörner *et al*<sup>[12]</sup> reported that the degree of IBL in colon cancer influenced long-term survival. In their study, blood loss of 250 mL or more during surgery was a risk factor for overall mortality in both univariate and multivariate analyses. Nagai *et al*<sup>[13]</sup> demonstrated that IBL greater than 2000 mL was related to poor prognosis in patients with pancreatic cancer. These authors suggested that successful curative resection with limited blood loss can contribute to improved survival. With regard to gastric cancer, few studies have focused on IBL. Dhar *et al*<sup>[10]</sup> reported that IBL more than 500 mL was an independent prognostic factor. Kamei *et al*<sup>[11]</sup> demonstrated that the cumulative survival rate was significantly lower in patients with IBL  $\geq$  475 mL than in patients with IBL < 475 mL ( $P = 0.0038$ ), and IBL was a critical risk factor for peritoneal recurrence after curative resection of advanced gastric cancer. Our data are consistent with those results and strongly suggest that IBL, rather than transfusion, was an independent prognostic factor for gastric cancer after curative resection.

In previous studies, blood loss of 475 or 500 mL was proposed as a threshold for prognostic significance<sup>[10,11]</sup>. To date, no study has conducted a detailed statistical analysis by classifying patients into groups based on the level of IBL during resection for gastric cancer. When the thresholds were set at 200 and 400 mL, the OS was significantly affected based on a comparison between these 3 groups. The 5-year OS rates were 51.2%, 39.4% and 23.4% for IBL < 200 mL, 200-400 mL and > 400 mL, respectively (< 200 mL *vs* 200-400 mL,  $P < 0.001$ ; 200-400 mL *vs* > 400 mL,  $P = 0.003$ ; < 200 mL *vs* > 400 mL,  $P < 0.001$ ). Even when deaths due to factors other than gastric cancer were excluded, the differences in cancer-specific survival among the three groups were still significant. This clearly demonstrated the negative influence of IBL on survival after curative gastrectomy. Pathological stage is assumed to be the most important prognostic factor for gastric cancer following curative gastrectomy. Therefore, we stratified patients by TNM stage. Even after stratification, the same trend, *i.e.*, better outcomes in patients with a small amount of IBL, was still observed in each stage. Thus, reducing IBL in resectable gastric cancer may provide further improvements in survival. According to the results of the present study, for patients staged TNM I and II, IBL should be controlled within 200 mL to achieve a better outcome. In patients staged TNM III, IBL should be no more than 400 mL.

Blood transfusion is needed when performing complex surgery with a large amount of IBL. Although many studies<sup>[17-21]</sup> have confirmed that perioperative blood transfusion leads to poor outcome in gastric cancer, some studies<sup>[22-26]</sup> do not support this. In the present study, perioperative transfusion was a prognostic factor, but not an independent prognostic factor in the multivariate analysis. When the influence of IBL was excluded, OS did not dif-

fer significantly between patients with and without transfusion, although 5-year OS was higher in patients without transfusion than in patients with transfusion if the IBL was similar. However, when excluding the influence of transfusion, patients whose IBL was less than 200 mL had significantly better survival than those with IBL of 200 mL or more. The effect of IBL on survival was more pronounced than that of red blood transfusion.

It is still unclear why IBL affects the long-term outcome of patients. It is thought that excessive IBL reduces the body's immunity and thus its ability to fight cancer cells<sup>[10]</sup>. In a study conducted by Bruns *et al*<sup>[27]</sup>, IBL more than 700 mL following gastrointestinal surgery was associated with a significant decrease in natural killer cell activity, producing an unfavorable effect on patient survival. However, the degree of immune suppression was not assessed in this study. This should be examined in a future trial to clarify whether patients with excessive IBL have severe immune suppression resulting in a poor overall survival rate. Another possible explanation is that IBL is associated with peritoneal recurrence which leads to poor survival. It has been reported that operative blood loss is an independent risk factor for peritoneal recurrence of curatively resectable advanced gastric cancer<sup>[11]</sup>. In open abdominal surgery, most operative blood loss accumulates in the abdominal cavity, and thus, the peritoneal surface is considered to have direct contact with blood components. As extravascular blood cells, such as leukocytes and platelets, are activated, they may produce a number of soluble factors that may produce a favorable microenvironment for malignant cells. In fact, activated neutrophils, macrophages, and platelets are capable of producing a large amount of angiogenic factors, such as vascular endothelial growth factor, on the peritoneal surface, which is critical for the survival of isolated cancer cells<sup>[28,29]</sup>. Unfortunately, recurrence data was not obtained in our study.

IBL has been shown to be correlated with postoperative complications<sup>[30]</sup>. In the present study, the incidence of postoperative complications increased when the amount of IBL was high. Previous studies have affirmed the negative influence of postoperative complications on survival for many malignancies<sup>[31-35]</sup>. Sierzega *et al*<sup>[7]</sup> reported that anastomotic leakage was an independent prognostic factor for gastric adenocarcinoma following total gastrectomy. Tokunaga *et al*<sup>[35]</sup> found that postoperative intra-abdominal infectious complications had an adverse effect on 5-year OS and relapse-free survival rate. Our results were in accordance with those reports and showed that the presence of postoperative complications was an independent prognostic factor for OS. As a higher rate of complications was associated with a larger amount of IBL, we consider that the difference in the incidence of postoperative complications among the three groups was a possible contributing factor to the survival difference among the three groups.

As IBL is an independent prognostic factor and patients with IBL less than 200 mL had the best outcome,

it is necessary to explore the potential factors influencing IBL and to develop new surgical methods to reduce IBL. It is obvious that IBL could be affected by the type of gastrectomy and combined organ resection. Patients with tumors located in the upper 1/3 or more than 2/3 the area usually undergo a total gastrectomy or combined spleen resection, which may result in a larger amount of IBL. Lymph node dissection is considered to be a complex procedure and can easily lead to bleeding, especially dissection of the lymph nodes around the celiac trunk. We have used an ultrasonic scalpel for lymph node dissection of gastric cancer since 2006. Ultrasonic surgical devices have been reported to provide advantages in terms of operative time and blood loss<sup>[36,37]</sup>. A study conducted by Inoue K and colleagues showed that blood loss was significantly lower in patients using ultrasonic scalpel than in those not using the ultrasonic scalpel (median 351.0 mL *vs* 569.5 mL;  $P = 0.016$ )<sup>[38]</sup>. From this point of view, it is actually the application of the ultrasonic scalpel that leads to reduced IBL rather than the year, although year of surgery was found to be an independent risk factor for IBL in the present study.

In conclusion, IBL was found to be an independent prognostic factor for gastric cancer after curative resection. It can be used to stratify the risk for gastric cancer prognosis. Meticulous surgery is needed and new methods should be considered to decrease the amount of IBL and improve the long-term outcome of patients following curative gastrectomy.

## COMMENTS

### Background

Intraoperative blood loss (IBL) has been shown to be associated with poor outcome in various types of malignancy. However, the relationship between the amount of IBL and outcome of gastric cancer is still unclear.

### Research frontiers

IBL can not be avoided in surgery. Excessive blood loss may result in more postoperative complications and poorer prognosis. Research has shown the negative association between IBL and prognosis of many malignancies. Few researchers have focused on IBL during resection of gastric cancer. In this study, the authors demonstrated that IBL was an independent prognostic factor for gastric cancer after curative resection.

### Innovations and breakthroughs

Many studies have affirmed that perioperative blood transfusion leads to poor outcome in gastric cancer. However, when performing complex surgery, blood transfusion is required due to a large amount of IBL, which was also reported to have an adverse effect on survival. The impact of IBL on survival may be confused by blood transfusion. This study evaluated the prognostic value of both factors on survival in gastric cancer patients after curative resection and found that IBL influenced the prognosis of gastric cancer rather than blood transfusion.

### Applications

By understanding the negative association between the amount of IBL and prognosis of gastric cancer, this study may stimulate surgeons to pay attention to decreasing the amount of IBL during curative gastrectomy.

### Terminology

IBL is the amount of blood loss during surgery which is visually estimated by anesthesiologists immediately after surgery. Extranodal metastasis was defined as the presence of tumor cells in extramural soft tissue that was discontinuous with either the primary lesion or locoregional lymph nodes.

### Peer review

The IBL and perioperative transfusion have been the topics concerned by sur-

geons. And IBL has been shown to be associated with poor outcome in various types of malignancy. This study shows that IBL is an independent prognostic factor for gastric cancer patients after curative resection. This conclusion has some significance for guiding clinical work.

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## Effects of extended lymphadenectomy and postoperative chemotherapy on node-negative gastric cancer

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### Abstract

**AIM:** To investigate the effects of extended lymphadenectomy and postoperative chemotherapy on gastric cancer without lymph node metastasis.

**METHODS:** Clinical data of 311 node-negative gastric cancer patients who underwent potentially curative gastrectomy with more than 15 lymph nodes resected, from January 2002 to December 2006, were analyzed retrospectively. Patients with pT4 stage or distant metastasis were excluded. We analyzed the relationship between the D2 lymphadenectomy and the 5-year survival rate among different subgroups stratified by clinical features, such as age, tumor size, tumor location and depth of invasion. At the same time, the relationship between postoperative chemotherapy and the 5-year survival rate among different subgroups were also analyzed.

**RESULTS:** The overall 5-year survival rate of the entire

cohort was 63.7%. The 5-year survival rate was poor in those patients who were: (1) more than 65 years old; (2) with tumor size larger than 4 cm; (3) with tumor located in the upper portion of the stomach; and (4) with pT3 tumor. The survival rate was improved significantly by extended lymphadenectomy only in patients with pT3 tumor ( $P = 0.019$ ), but not in other subgroups. Moreover, there was no significant difference in survival rate between patients with and without postoperative chemotherapy among all of the subgroups ( $P > 0.05$ ).

**CONCLUSION:** For gastric cancer patients without lymph node metastasis, extended lymphadenectomy could improve the survival rate of those who have pT3-stage tumor. However, there was no evidence of a survival benefit from postoperative chemotherapy alone.

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**Key words:** Gastric cancer; Lymph node negative metastasis; Extended lymphadenectomy; D2 lymphadenectomy; Chemotherapy

**Core tip:** Little information is available regarding the effects of D2 lymphadenectomy and postoperative chemotherapy in patients with node-negative early gastric cancer. Data of 311 gastric cancer patients without lymph node metastasis were analyzed retrospectively. Results showed that D2 lymphadenectomy could improve the survival rate of patients with pT3-stage tumor. However, there was no evidence of a survival benefit from postoperative chemotherapy. In conclusion, it is recommended that D2 lymphadenectomy with gastrectomy be applied for node-negative patients with pT3 gastric cancer whereas the effects of postoperative chemotherapy in patients with node-negative early gastric cancer need to be further studied.

Xue Q, Wang XN, Deng JY, Zhang RP, Liang H. Effects of extended lymphadenectomy and postoperative chemotherapy

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## INTRODUCTION

Gastric cancer is one of the most common malignancies worldwide, with a high mortality rate<sup>[1]</sup>. Many studies indicate that, in gastric cancer, the presence or absence of lymph node metastasis is an important prognostic factor that could influence the prognosis of patients following curative gastrectomy<sup>[2-5]</sup>. It has been shown that an extended (D2) lymphadenectomy could bring benefits to the long-term survival rate of patients with node-positive gastric cancer<sup>[6,7]</sup>, and D2 lymphadenectomy has become a standard surgical procedure for curative treatment in South Korea and Japan<sup>[8]</sup>. However, recurrence and metastasis are also noted in node-negative gastric cancer after curative resection, and there are few studies on the effects of D2 lymphadenectomy in patients with node-negative gastric cancer. At the same time, postoperative chemotherapy is considered an effective treatment option for patients with advanced gastric cancer<sup>[9-11]</sup>, nevertheless, whether it could bring benefit to node-negative gastric cancer patients who received curative gastrectomy still needs to be further elucidated. Hence, the aim of this study was to investigate whether extended lymphadenectomy and postoperative chemotherapy could bring a survival benefit to patients with node-negative gastric cancer.

## MATERIALS AND METHODS

Between January 2002 and December 2006, 867 patients diagnosed with gastric adenocarcinoma were treated with curative gastrectomy (R0 resection) and with more than 15 lymph nodes resected at the Department of Gastric Cancer Surgery, Tianjin Medical University Cancer Hospital and City Key Laboratory of Cancer Prevention and Therapy, Tianjin, China. Of these patients, 311 had lymph node-negative metastasis. There were 230 males and 81 females with ages ranging from 21 to 82 years (60.0 ± 11.2 years). Patients with pT4 stage or distant metastasis were excluded. D2 lymphadenectomy was performed according to the guidelines of lymph node stations defined by the Japanese Gastric Cancer Association<sup>[12]</sup>.

Patients were stratified according to clinical features including age, sex, tumor size, location, Borrmann type, depth of invasion, and pathologic examination. Furthermore, patients with poor prognosis were stratified into subgroups according to the number of resected lymph nodes (LNs) and whether they received postoperative chemotherapy. According to the number of resected LNs, patients were divided into a 15-24 subgroup and a ≥ 25 subgroup. Patients were also divided into groups according to whether or not they received postoperative chemotherapy.

**Table 1 Clinicopathologic factors of patients with node-negative gastric cancer**

Characteristics	n	5-yr survival rate	χ <sup>2</sup>	P value
Gender			1.416	0.234
Male	230	67.40%		
Female	81	72.80%		
Age (yr)			4.979	0.026
< 65	156	75.20%		
≥ 65	155	62.40%		
Tumor size (cm)			5.930	0.015
≤ 4	166	73.80%		
> 4	145	63.00%		
Tumor location			8.721	0.033
Upper	103	58.70%		
Middle	45	67.90%		
Lower	150	76.50%		
Total	13	68.40%		
Borrmann type			3.834	0.280
I	60	71.60%		
II	129	74.20%		
III	108	62.80%		
IV	14	57.10%		
Depth of invasion			13.676	0.001
T1	22	100.00%		
T2	69	78.40%		
T3	220	62.20%		
Pathology			2.689	0.101
Differentiated	124	73.90%		
Undifferentiated	187	65.30%		

Patients received postoperative chemotherapy (FOLF-OX6): oxaliplatin (100 mg/m<sup>2</sup>) and leucovorin (400 mg/m<sup>2</sup>), followed by 5-FU (400 mg/m<sup>2</sup>) bolus, then a 46 h continuous infusion of 5-FU (3000 mg/m<sup>2</sup>). The regimen was repeated every 2 wk for 6-8 cycles and follow-up was conducted until November 2011 or until death. Data collection was based on review of clinical charts and on telephone interviews with discharged patients.

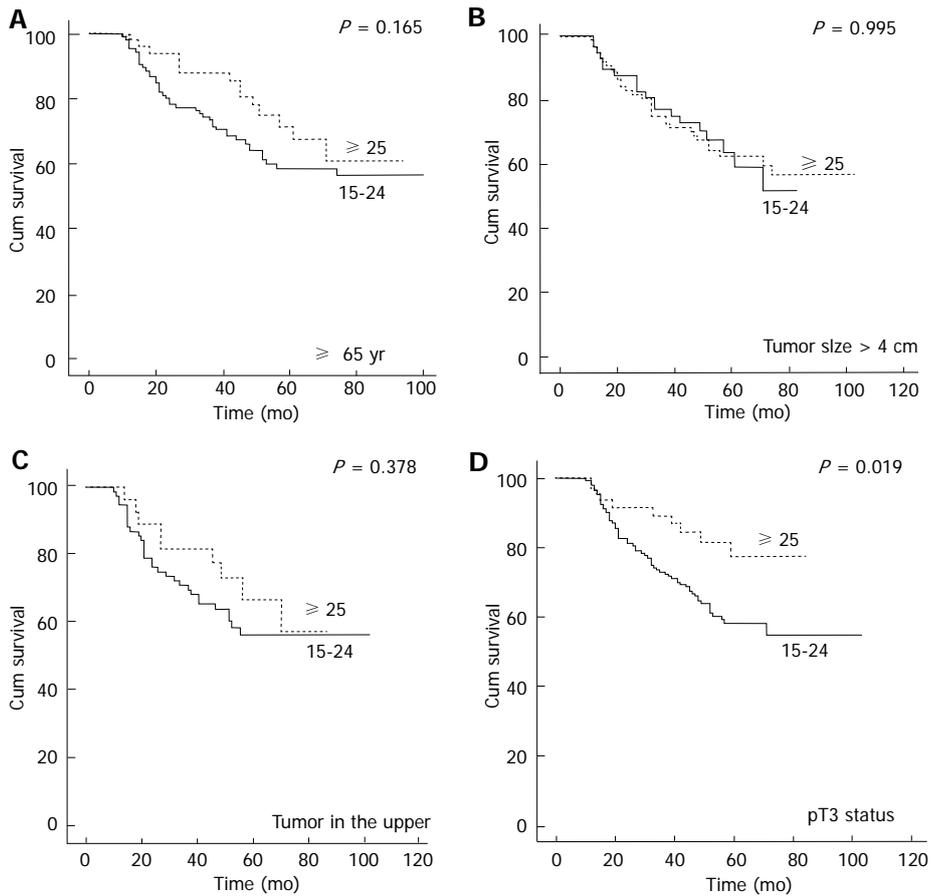
### Statistical analysis

The analysis was performed using the Statistical Package for Social Science (SPSS), version 13.0 for Windows. Actuarial survival rate was determined *via* the Kaplan-Meier method, and univariate comparisons of survival between different groups were performed using the log rank test. Significance of differences was accepted at P value < 0.05.

## RESULTS

The overall 5-year survival rate (5-YSR) of the entire cohort was 63.7%. Factors influencing the 5-YSR were as follows: age (P = 0.026), tumor size (P = 0.015), tumor location (P = 0.033) and depth of invasion (P < 0.001). The survival rate was lower in patients who were more than 65 years old, with tumor size larger than 4 cm, with tumor located in the upper portion of the stomach, or with pT3 status. Gender (P = 0.234), Borrmann type (P = 0.280) and pathological types (P = 0.101) had no significant influence on the survival rate. The clinicopathological variables tested in the univariate analysis are shown in Table 1.

The survival rate of different groups divided by the



**Figure 1** Five-year survival curve for patients with N0 gastric cancer according to the number of resected lymph nodes. A: In  $\geq 65$  years group, survival curve for 155 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ); B: In tumor size  $> 4$  cm group, survival curve for 145 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ); C: In the upper location group, survival curve for 103 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ); D: In pT3 group, survival curve for 220 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ).

**Table 2** Major postoperative complications observed in the study

Type of complications	15-24 LNs removed (n = 189)	Above 25 LNs removed (n = 122)	$\chi^2$	P value
Pulmonary	16	13		
Abdominal abscess	15	9		
Pancreatic fistula	5	3		
Anastomotic leak	2	2		
Lymphorrhhea	4	3		
Paralytic ileus	2	2		
Others	3	2		
Total	47	34	0.347	0.556

LNs: Lymph nodes.

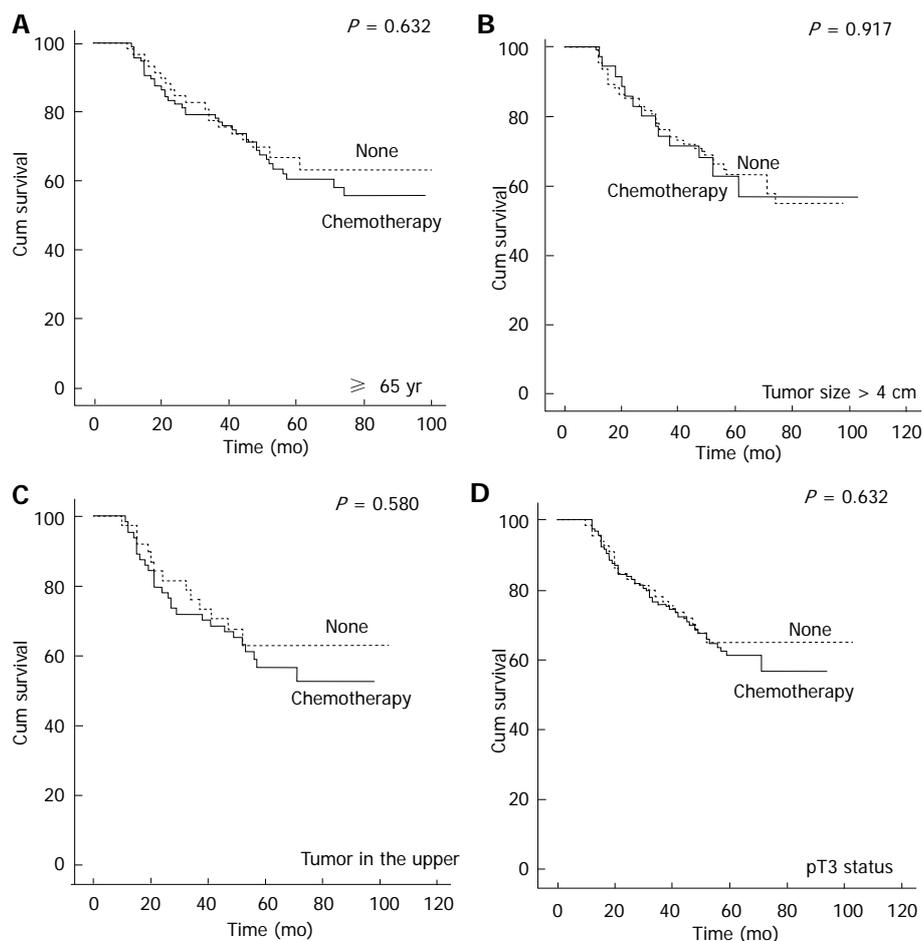
number of resected LNs and whether patients received post-operative chemotherapy were compared between groups stratified by age, tumor size, tumor location and pT status. In patients who were more than 65 years old, with tumor size larger than 4 cm, with tumor located in the upper portion of the stomach, the survival rate was not significantly different between the two subgroups of patients with 15-24 and  $\geq 25$  LNs dissected ( $P = 0.165, 0.995, 0.378$ , respectively). However, for patients with pT3 cancer, the survival rate in patients with  $\geq 25$  LNs dissected was significantly higher than that of patients with 15-24 LNs dissected ( $P = 0.019$ ). The survival curves are presented in Figure 1.

There was no significant difference in survival rates between patients with or without postoperative chemotherapy in all 4 groups, divided according to whether patients were more than 65 years old, with tumor size larger than 4 cm, with tumor located in the upper portion of the stomach or in pT3 status ( $P = 0.632, 0.917, 0.580, 0.632$ , respectively). The survival curves are shown in Figure 2.

Eighty-one of the 311 patients developed postoperative general and surgical complications (morbidity: 26.0%), such as pulmonary affections, abdominal abscess, pancreatic fistula, anastomotic leak, lymphorrhhea, paralytic ileus, and no patients died during the perioperative period. Forty-seven patients with complications were in the patient group with 15-24 LNs dissected, and thirty-four were in the group with  $\geq 25$  LNs dissected. There was no significant difference in the post-operative complication rate between these two groups ( $P = 0.556$ ). Table 2 lists the type of complications and their frequency.

## DISCUSSION

Nowadays, due to the significant improvements in diagnosing techniques as well as the popularization of health screening, gastric cancers tend to be detected in their early stages. Of all the patients with gastric cancer treated in our hospital, 35.9% were in the early period. It is commonly considered that lymph node metastases is one of the most important prognostic factors for patients with gastric cancer after curative surgery<sup>[13]</sup>. What's more, re-



**Figure 2** Five-year survival curve for patients with N0 gastric cancer according to whether patients received postoperative chemotherapy. A: In  $\geq 65$  years group, survival curve for 155 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy; B: In tumor size  $> 4$  cm group, survival curve for 145 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy; C: In the upper location group, survival curve for 103 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy; D: In pT3 group, survival curve for 220 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy.

currence and metastasis were also noted in gastric cancer without lymph node metastasis after curative resection. The recurrence rate of early gastric cancer (EGC) was reported as 1.7%-3.4%<sup>[14-17]</sup>. In previous studies<sup>[18-20]</sup>, it was reported that some variables such as pT status, tumor size, tumor location, Lauren type and the number of resected LNs were associated with survival in pN0 gastric cancer. According to our study, the survival rate was lower in patients whose age was more than 65 years old, tumor size was larger than 4 cm, tumor location was in the upper portion of the stomach, or tumor stage was pT3.

Studies have shown that D2 lymphadenectomy could improve the overall survival of patients with advanced node-positive gastric cancer<sup>[21,22]</sup>. D2 lymphadenectomy for pN0 gastric cancer patients who received gastrectomy has been a topic of much discussion. Some recent studies reported that D2 lymphadenectomy with gastrectomy could prolong the survival rate of patients with node-negative advanced gastric cancer<sup>[23-25]</sup>. Consistently, in this study we found that the survival rate of node-negative patients with pT3 gastric cancer could be improved by D2 lymphadenectomy ( $P = 0.019$ ). One possible reason is that the node and tissue with micrometastasis were removed by D2 lymphadenectomy. In one recent study<sup>[26]</sup> it is reported that lymph node micro-metastasis was detectable in 10% of node-negative EGC patients, and occurred more frequently in cases with larger tumor,

lymphatic invasion, or venous invasion. Based on these results, it is recommended that, for node-negative patients diagnosed with pT3 gastric cancer by endoscopic ultrasound preoperatively or at operation, the D2 lymphadenectomy should be performed even without clinically detectable node metastases. However, for other patients with poor survival rate, the effect of D2 lymphadenectomy is inconspicuous.

Previously, it was claimed that the postoperative morbidity and mortality may be increased by D2 lymphadenectomy<sup>[27,28]</sup>. However, with the improvement of surgical techniques, this situation has been changed. As reported in one study<sup>[29]</sup>, there was no difference in the incidence of four major complications (anastomotic leak, pancreatic fistula, abdominal abscess, pneumonia) between the D2 group and D2 plus group. In this study, we also found that the mortality of postoperative general complications was not significantly different between two groups with and without D2 lymphadenectomy (24.9% *vs* 27.9%,  $P = 0.556$ ).

To date, it has been recommended that postoperative chemotherapy should be used in advanced gastric cancer<sup>[9-11,30,31]</sup>. The efficacy and safety of FOXFOL6 regimen for advanced gastric cancer has been demonstrated by a phase II study<sup>[32]</sup>. However, the therapeutic value of chemotherapy for pN0 gastric cancer is still unclear and scarcely reported. Inconsistent with results

from advanced gastric cancer, we found that the survival rate of pN0 gastric cancer patients with postoperative chemotherapy was not significantly different from that of patients without chemotherapy, regardless of whether patients were more than 65 years old ( $P = 0.632$ ), with tumor size larger than 4 cm ( $P = 0.917$ ), with tumor located in the upper portion of the stomach ( $P = 0.580$ ) or in pT3 status ( $P = 0.632$ ).

There were several limitations to the current study. First, in this study, the overall survival is evaluated as an endpoint, while disease-free or recurrence-free survival was not investigated, which are also important for patients with gastric cancer. Second, the extent of lymphadenectomy was variable according to the decisions made by different surgeons, which may affect the results of this study. Finally, as this is a retrospective study, the regimen and dose of chemotherapy might be multifarious, which may affect the accuracy of the comparison of groups.

In conclusion, it is recommended that D2 lymphadenectomy with gastrectomy be applied for node-negative patients with pT3 gastric cancer. However, the effect of postoperative chemotherapy in pN0 gastric cancer patients still need to be further studied.

## COMMENTS

### Background

Many studies have shown that D2 lymphadenectomy could bring benefits to the long-term survival rate of patients with node-positive gastric cancer, however, little information is available regarding its effects in patients with node-negative gastric cancer. At the same time, although the efficacy and safety of FOXFOL6 regimen for advanced gastric cancer has been validated by many studies, the effects of postoperative chemotherapy for pN0 gastric cancer are still unclear and scarcely reported.

### Research frontiers

Some recent studies reported that D2 lymphadenectomy with gastrectomy could prolong the survival rate of patients with node-negative gastric cancer, whereas the impact of postoperative chemotherapy on the survival is scarcely reported.

### Innovations and breakthroughs

The authors retrospectively reviewed 311 patients with node-negative gastric cancer, who were treated with curative gastrectomy and with more than 15 lymph nodes resected at a hospital in Tianjin between 2002 and 2006, to assess whether D2 lymphadenectomy and postoperative chemotherapy may affect their survival rate.

### Applications

The authors suggest that, for node-negative patients diagnosed with pT3 gastric cancer, D2 lymphadenectomy be performed even without clinically detectable node metastases. However, for other patients with poor survival rate, the effect of D2 lymphadenectomy is less obvious.

### Peer review

This article demonstrated the necessity of extended lymphadenectomy for gastric cancer patients without lymph node metastasis. In this study, the authors found that the survival rate of node-negative patients with pT3 gastric cancer could be improved by D2 lymphadenectomy.

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## Hypermethylation of *TGF-β1* gene promoter in gastric cancer

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### Abstract

**AIM:** To examine transforming growth factor- $\beta 1$  (*TGF-β1*) promoter methylation in gastric cancer and to determine if *Helicobacter pylori* (*H. pylori*) or interleukin (IL)-1 $\beta$  could induce *TGF-β1* hypermethylation *in vitro*.

**METHODS:** We examined the frequency and extent of *TGF-β1* promoter methylation using methylation-specific PCR in the gastric tissues from 47 gastric cancer patients and 39 non-gastric cancer subjects. *H. pylori* infection was confirmed by a positive result from either a serological test, histological analysis or C<sup>13</sup> urea breath test. GES-1 and MKN-45 cells co-cultured with *H. pylori* or treated with IL-1 $\beta$  for 12, 24 and 48 h *in vitro* tested the effects of *H. pylori* or IL-1 $\beta$  on *TGF-β1*.

**RESULTS:** Twenty-four/forty-seven (51%) cases of gastric cancer (GC) tissues showed *TGF-β1* promoter methylation, 15/47 (31.9%) cases of matched non-cancerous gastric mucosa tissues from the GC patients, and 11/39 (28%) case of the normal gastric mucosa tissues from non-GC subjects showed *TGF-β1* promoter methylation (51% vs 28%,  $P < 0.05$ ). Significantly higher levels of methylation of *TGF-β1* were found in the tumor tissues than in non-tumor tissues from GC patients ( $0.24 \pm 0.06$  vs  $0.17 \pm 0.04$ ,  $P < 0.05$ ) and normal gastric tissues from non-GC subjects ( $0.24 \pm 0.06$  vs  $0.15 \pm 0.03$ ,  $P < 0.05$ ). *TGF-β1* methylation was found in 48.3% of *H. pylori*-positive gastric mucosal tissues whereas only 23.1% of *H. pylori*-negative gastric mucosal tissues showed *TGF-β1* methylation (48.3% vs 23.1%,  $P < 0.05$ ). IL-1 $\beta$  appeared to induce a dose-dependent methylation of *TGF-β1* and the strongest methylation was observed in GES-1 cells treated with 2.5 ng/mL of IL-1 $\beta$  for 48 h. Further studies showed that pre-treatment of GES-1 cells with 20 ng/mL IL-1RA for 1 h could partially abolish the effect of IL-1 $\beta$  on *TGF-β1* methylation. Infection of GES-1 cells by *H. pylori* was not found to induce significant *TGF-β1* promoter methylation.

**CONCLUSION:** Our data revealed that *TGF-β1* promoter is methylated in GC patients. IL-1 $\beta$  may be an important mediator for *H. pylori* induced gene methylation during GC development.

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**Key words:** Transforming growth factor- $\beta$ 1; Interleukin-1 $\beta$ ; Methylation; *Helicobacter pylori*; Gastric cancer

**Core tip:** *In vitro* studies showed that GES-1 cells exposed to *Helicobacter pylori* (*H. pylori*) did not show significant transforming growth factor- $\beta$ 1 (*TGF- $\beta$ 1*) methylation. However, treatment of the GES-1 cells with interleukin (IL)-1 $\beta$  led to a dose-dependent methylation of *TGF- $\beta$ 1*, which was partially abolished by IL-1RA. The high levels of *TGF- $\beta$ 1* promoter methylation in *H. pylori* positive patients was likely the result of *H. pylori*-induced inflammation rather than *H. pylori* itself. IL-1 $\beta$  may be an important mediator for *H. pylori*-induced gene methylation during gastric cancer development.

Wang YQ, Li YM, Li X, Liu T, Liu XK, Zhang JQ, Guo JW, Guo LY, Qiao L. Hypermethylation of *TGF- $\beta$ 1* gene promoter in gastric cancer. *World J Gastroenterol* 2013; 19(33): 5557-5564 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5557.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5557>

## INTRODUCTION

Genetic and epigenetic alterations in tumor suppressor genes or oncogenes are implicated in cancer formation. DNA methylation is the major form of epigenetic change in eukaryotic genomes. It involves the addition of a methyl group to the carbon 5 position of the cytosine ring within the CpG dinucleotide. CpG islands (CGIs) are regions of the genome that contain a large number of CpG dinucleotide repeats. In mammalian genomes, CGIs usually extend for 300-3000 base pairs. They are located within, and close to, sites of about 40% of gene promoters. It is estimated that in mammalian genomes, about 80% of CpG dinucleotides are methylated. However, CpG dinucleotides in regions abundant in GC pairs, such as CGIs, are normally protected from DNA methylation, and this is an important controlling mechanism for gene promoters and gene expression<sup>[1]</sup>. Although most CGIs linked to promoters are non-methylated, recent studies have revealed that promoter CpG hypermethylation, associated with transcriptional inactivation, may play a pivotal role in tumorigenesis<sup>[2]</sup>.

Gastric cancer (GC) remains a major health threat because of its high incidence, poor prognosis and limited treatment options. Multiple epigenetic and genetic alterations have been identified in GC patients. High levels of aberrant CpG island methylation and DNA methylation in the gastric mucosae correlate with increased GC risk, and as such, they are increasingly recognized as candidate markers for GC<sup>[3]</sup>.

Clinical and epidemiological studies have demonstrated that *Helicobacter pylori* (*H. pylori*) infection is correlated strongly with aberrant methylation in GC, whereas eradication of *H. pylori* significantly reduces gene methylation<sup>[4,5]</sup>.

Clearly, *H. Pylori*-induced aberrant methylation plays a role in GC formation. However, the precise molecular mechanisms of how *H. Pylori* might induce aberrant CpG island methylation remain elusive.

Chronic inflammation is a well-known promoting factor for many cancers. Approximately 15%-20% of all human cancers are related to chronic inflammation<sup>[6]</sup>. GC is a typical inflammation-related malignancy, being closely linked to *H. Pylori*-induced chronic inflammation in gastric mucosa. Chronic inflammation in the esophagus and colon may precipitate aberrant methylation, but whether *H. pylori* itself or the chronic inflammation caused by *H. pylori* infection induces methylation in CGIs remains controversial<sup>[7,8]</sup>.

*H. pylori* infection is characterized by infiltration of inflammatory cells, such as neutrophils and lymphocytes, into the gastric mucosa, as well as increased production of inflammatory cytokines<sup>[9,10]</sup>. Interleukin (IL)-1 $\beta$  is a pro-inflammatory cytokine primarily secreted by activated monocytes/macrophages in response to bacterial infection. IL-1 $\beta$  mediates many pathophysiological events during host-environment interactions. Recent studies have demonstrated that the levels of several inflammatory cytokines including IL-1 $\beta$ , IL-6, IL-8 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) are significantly higher in the gastric mucosal tissues from *H. pylori*-positive patients than those from *H. pylori*-negative patients<sup>[11-13]</sup>. It was further demonstrated that IL-1 $\beta$  could directly induce promoter methylation of *E-cadherin*, an important extracellular matrix component involved in the maintenance of epithelial stability: *H. pylori*-induced methylation of *E-cadherin* promoter was mediated through IL-1 $\beta$ <sup>[14]</sup>.

Transforming growth factor- $\beta$ 1 (*TGF- $\beta$ 1*) is an anti-inflammatory cytokine with multiple, and perhaps even opposite, biological effects in many tissues. TGF- $\beta$ 1 was shown to inhibit the growth of epithelial cells, but stimulates the proliferation of mesenchymal cells<sup>[15,16]</sup>. Many studies have shown that TGF- $\beta$ 1 is overexpressed in epithelial cancers and exerts its transforming potential through several mechanisms, such as stimulating the progression of stromal cells, promoting angiogenesis and suppressing immune surveillance<sup>[17]</sup>. However, TGF- $\beta$ 1 was reported to function as a tumor suppressor, because it could inhibit potently the proliferation of many types of cancer cells derived from breast, prostate, lung, colon and liver<sup>[18,19]</sup>. Furthermore, methylation-induced silencing of *TGF- $\beta$ 1* has been implicated in the development of several solid tumors, and silencing of *TGF- $\beta$ 1* signaling through methylation of the gene encoding its receptor have been reported<sup>[20,21]</sup>.

It has been reported that the expression level of host TGF- $\beta$ 1 in gastric mucosa was an important determinant for the pathogenesis of *H. pylori*-associated gastric diseases<sup>[22-24]</sup>. The gastric mucosa of *TGF- $\beta$ 1* null mice exhibit similar changes to those observed in *H. pylori*-associated gastritis, and these mice were found to progressively develop inflammatory diseases and die within 3-4 wk of birth<sup>[25]</sup>. However, the role of *TGF- $\beta$ 1* methylation in the development of *H. pylori*-related GC remains largely unknown.

In this study, we aimed investigate if *H. pylori* could induce promoter methylation of TGF- $\beta$ 1 in GC and whether IL-1 $\beta$  plays a role in this process.

## MATERIALS AND METHODS

### Patients and specimens

This study involved 47 consecutive GC patients (35 males and 12 females, mean age 56.2 years) who underwent gastrectomy and 39 consecutive non-GC subjects (28 males and 11 females, mean age 52.1 years) who underwent upper gastroduodenoscopy. *H. pylori* infection was confirmed by a positive result from either one of the following diagnostic approaches: serological test, histological analysis or C<sup>15</sup> urea breath test. No patients received prior *H. pylori* eradication therapy. Ninety-four gastric specimens (two from each patient, from tumor and non-tumor gastric mucosa) from GC patients and 39 gastric mucosa tissues from non-GC subjects were collected. The tissues were snap-frozen in liquid nitrogen and subsequently stored at -80 °C for the studies described below. Patients who received eradication therapy for *H. pylori* before the study and those with severe systemic diseases (such as major organ failure, server infection, autoimmune disease, organ transplantation and immunosuppressive therapy) were excluded.

### Cell culture

Human gastric epithelium cell line GES-1 was kindly provided by Professor Bingdong Zhu (School of Basic Medical Sciences, Lanzhou University). Cells were cultured in DMEM medium containing 10% FBS supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Bacterial strain and conditions

NCTC11637, a *CagA*-positive strain of *H. pylori*, was purchased from the American Type Cell Culture (ATCC) (Rockville, MI, United States). *H. pylori* were cultured on 4.2% Columbia Blood A gar (Youkang Foundation of Biological Science and Technology Beijing Co. Ltd. Beijing, China) containing 7.5% normal sheep blood and 0.5% antibiotics (vancomycin, 10 mg/mL; polymyxin, 0.025 mg/mL; and amphotericin B, 10 mg/mL) under micro-aerophilic conditions for 72 h. Bacteria were harvested and re-suspended in sterile phosphate buffered saline (PBS) and counted by absorbance at 660 nm (1 OD<sub>660</sub> = 1 × 10<sup>8</sup> colony forming units/mL).

### Infection of gastric epithelial cells by *H. pylori*

To establish an *in vitro* model of *H. pylori* infected gastric mucosa, GES-1 cells were grown to 80% confluence under the above-mentioned conditions. Cells were infected with live *H. pylori* at *H. pylori*/cell ratios of 5:1, 10:1, 50:1, and 100:1. To determine the involvement of IL-1 $\beta$  in *H. pylori*-induced pathology, GES-1 cells were pre-treated with human interleukin-1 receptor antagonist (IL-1RA) (Peptotech, Rocky Hill, NJ, United States) for 1 h before

*H. pylori* infection. IL-1RA was used at various concentrations (10, 20, 50 and 100 ng/mL) to select the concentration at which it can effectively block the IL-1 $\beta$  signaling. 20 ng/mL for 48 h was found to be an effective dose. All cells were cultured in 6-well plates at 37 °C in a humidified atmosphere for 12, 24 and 48 h.

### Treatment of GES-1 cells with IL-1 $\beta$

GES-1 cells were pre-treated with or without various concentrations of IL-1RA (10, 20, 50 and 100 ng/mL) for 1 h, followed by treatment with different concentrations IL-1 $\beta$  (Peptotech, Rocky Hill, NJ, United States) (0.1, 0.25, 1.0 and 2.5 ng/mL) for 12, 24, and 48 h in fresh serum-free DMEM medium.

### Methylation-specific polymerase chain reaction

Genomic DNA was extracted from each sample using the TIANamp Genomic DNA Kit (Tiangen Biotech Beijing Co. Ltd, Beijing, China), according to the manufacturer's instructions. The extracted DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Los Angeles, CA, United States), according to the manufacturer's instructions. After bisulfite treatment, DNA was purified using a Zymo-Spin™ IC Column (Zymo Research, Los Angeles, CA, United States) and resuspended in 10  $\mu$ L of dilution buffer (M-Elution Buffer, Zymo Research, Los Angeles, CA, United States). DNA methylation of the TGF- $\beta$ 1 promoter was analyzed by methylation-specific polymerase chain reaction (MSPCR), using Zymo Taq™ PreMix (Zymo Research), according to the manufacturer's instructions. Briefly, the bisulfite-modified DNA (2  $\mu$ L) was amplified using specific primers for methylated and unmethylated sequences of TGF- $\beta$ 1. The primer sequences used in the study were as follows. For methylated TGF- $\beta$ 1, forward: TATATCGTTCGTAAAGTTATAGCGT, reverse: AACATAAAAAAACTAAACCACCGTC. For unmethylated TGF- $\beta$ 1, forward: ATTTATATTGTTTGTAAAGTTATAGTGT, and reverse: AACATAAAAAAACTAAACCACCATC. PCR was performed in a 50- $\mu$ L reaction system, which contains Zymo Taq™ PreMix (25  $\mu$ L), forward primer (10  $\mu$ mol/L) 4  $\mu$ L, reverse primer (10  $\mu$ mol/L) 4  $\mu$ L, DNA template (2  $\mu$ L), and double distilled water (15  $\mu$ L). The reactions were hot-started at 97 °C for 10 min, followed by 40 cycles of reactions (15 s at 95 °C, 35 s for annealing, and 30 s at 72 °C) and a final 7-min extension in a Thermal Cycler (Veriti, ABI Co., Foster, CA, United States). For positive controls, we used CpGenome Universal Methylated DNA (Intergen, New York, NY, United States). Five microliters of PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide, and imaged using a VersaDoc Imaging System (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States).

### Densitometric analysis of TGF- $\beta$ 1 methylation Levels

Quantity One software v4.62 (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States) was used to perform

**Table 1 Methylation frequency for transforming growth factor-β1 in gastric tissues**

Case ID	GC patients (n = 47)		Non-GC subjects (n = 39)	
	<i>H. pylori</i>	TGF-β1 methylation	<i>H. pylori</i>	TGF-β1 methylation
	Tumor	Non-tumor		
1	+	Methylated	Methylated	+
2	+			+
3	+	Methylated	Methylated	+
4	+	Methylated		+
5	+	Methylated		+
6	+			+
7	+			+
8	+			+
9	+	Methylated		+
10	+	Methylated	Methylated	+
11	+			+
12	+			+
13	+	Methylated		+
14	+			+
15	+	Methylated	Methylated	+
16	+			+
17	+			+
18	+	Methylated		+
19	+	Methylated	Methylated	+
20	+			+
21	+	Methylated	Methylated	+
22	+			+
23	+			-
24	+			-
25	+	Methylated		-
26	+	Methylated	Methylated	-
27	+			-
28	+			-
29	+	Methylated	Methylated	-
30	+	Methylated	Methylated	-
31	+	Methylated		-
32	+			-
33	+			-
34	+	Methylated	Methylated	-
35	+			-
36	+	Methylated		-
37	+	Methylated	Methylated	-
38	+	Methylated		-
39	-			-
40	-	Methylated	Methylated	-
41	-	Methylated	Methylated	-
42	-			-
43	-			-
44	-			-
45	-			-
46	-	Methylated	Methylated	-
47	-			-

GC: Gastric cancer; TGF-β1: Transforming growth factor-β1; *H. pylori*: *Helicobacter pylori*.

densitometric analyses of methylated and un-methylated bands of *TGF-β1*, and the results were presented as the mean of three independent experiments. The methylation levels were calculated as the ratio of the value of methylated band to methylated plus unmethylated bands.

**Statistical analysis**

The frequencies of promoter methylation were compared between the two groups by two-sided Fisher’s exact test or Pearson  $\chi^2$  test. Differences in methylation levels of *TGF-β1*

between two groups were examined by Student’s *t* test. A *P* value of < 0.05 was considered statistically significant.

**RESULTS**

**Methylation frequency and levels of TGF-β1 in GC and non-GC subjects**

As shown in Table 1, 24/47 (51%) cases of GC tissues showed *TGF-β1* promoter methylation, 15/47 (31.9%) cases of matched non-cancerous gastric mucosa tissues from the GC patients, and 11/39 (28%) cases of the normal gastric mucosa tissues from non-GC subjects showed *TGF-β1* promoter methylation (51% vs 28%, *P* = 0.032).

To evaluate the levels of *TGF-β1* methylation, we analyzed quantitatively the MSPCR product bands by their fluorescence intensities. Typical MSPCR bands are shown in Figure 1A, and the quantitative data for the methylation levels of *TGF-β1* in gastric tissues are shown in Figure 1B. Significantly higher levels of methylation of *TGF-β1* were found in the tumor tissues (0.24 ± 0.06) than in non-tumor tissues from GC patients (0.17 ± 0.04) (*P* = 0.001) and normal gastric tissues from non-GC subjects (0.15 ± 0.03) (*P* = 0.001).

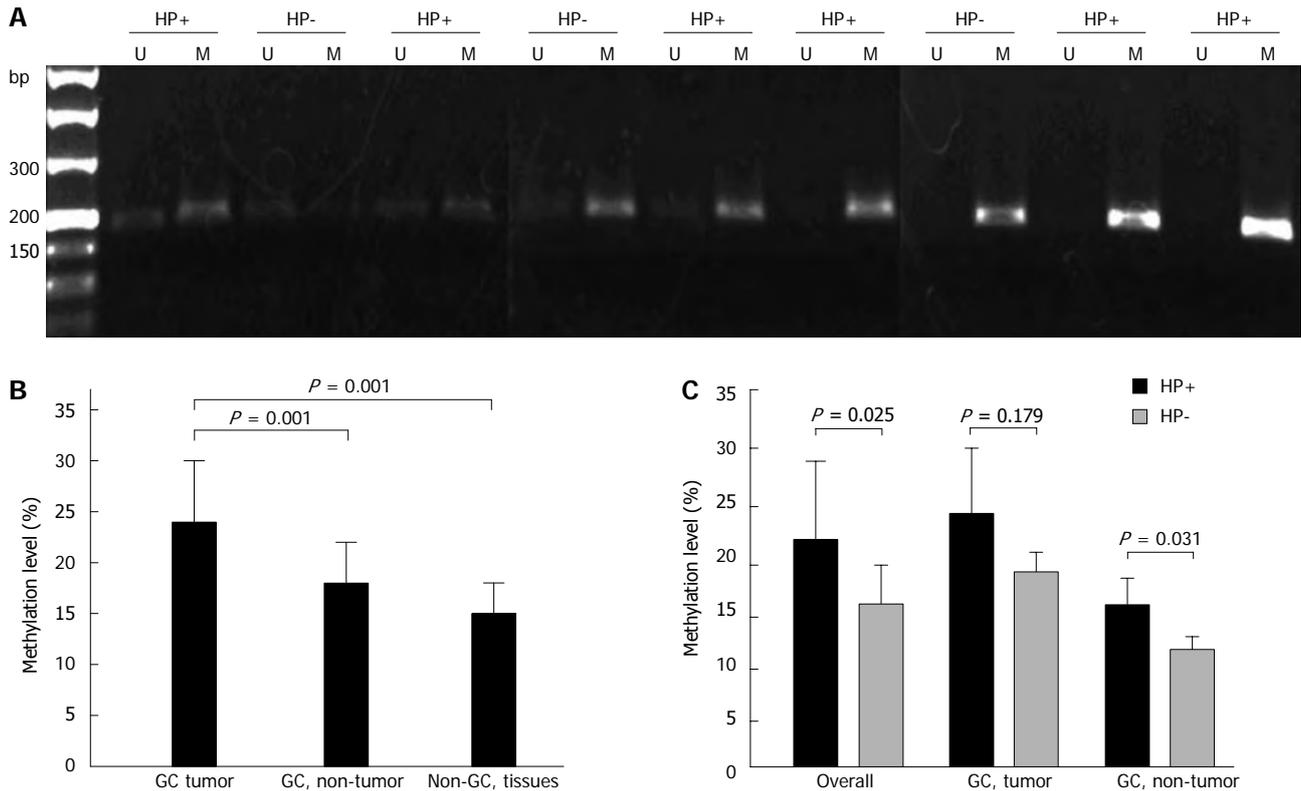
**Methylation frequency and levels of TGF-β1 in H. pylori-positive and H. pylori-negative subjects**

To examine the impact of *H. pylori* on the methylation of *TGF-β1*, we compared the methylation frequency of *TGF-β1* in *H. pylori*-positive and *H. pylori*-negative GC tissues and non-cancerous gastric tissues. As shown in Table 1 and best shown in Table 2, 21/38 (55.3%) cases of *H. pylori*-positive GC tissues showed *TGF-β1* methylation, whereas only 3/9 (33.3%) cases of *H. pylori*-negative GC tissues showed *TGF-β1* methylation. In non-cancerous gastric tissues obtained from GC patients, the frequency of *TGF-β1* methylation appeared to be similar between *H. pylori*-positive and *H. pylori*-negative gastric tissues (31.6% vs 33%, *P* = 0.919). In normal gastric mucosa from non-GC patients, *H. pylori*-positive tissues exhibited more frequent *TGF-β1* methylation than the *H. pylori*-negative gastric tissues (36.4% vs 17.6%, *P* = 0.288). Overall, *TGF-β1* methylation was found in 48.3% of *H. pylori*-positive gastric mucosal tissues, whereas only 23.1% of *H. pylori*-negative gastric mucosal tissues showed *TGF-β1* methylation (48.3% vs 23.1%, *P* = 0.029).

Further densitometric analysis showed that *H. pylori*-positive GC tumor tissues exhibited much higher levels of *TGF-β1* methylation than *H. pylori*-negative GC tumor tissues (Figure 1A and C). Furthermore, higher levels of *TGF-β1* methylation were also found in *H. pylori* positive non-GC mucosal tissues (Figure 1C). Overall, more *TGF-β1* methylation was present in *H. pylori* positive gastric mucosa than in *H. pylori*-negative gastric tissues (0.23 ± 0.06 vs 0.16 ± 0.03, *P* = 0.025) (Figure 1C).

**Effect of IL-1β signaling on TGF-β1 promoter methylation**

To examine the mechanisms of *TGF-β1* methylation, GES-1 cells were incubated in the presence or absence



**Figure 1** Detection of transforming growth factor-β1 promoter methylation by methylation-specific polymerase chain reaction. Genomic DNA was extracted from human gastric tissues. A: Representative methylation-specific polymerase chain reaction results from gastric cancer (GC) tissues with or without *Helicobacter pylori* (*H. pylori*) infection are shown; B: Levels of transforming growth factor-β1 (*TGF-β1*) promoter methylation in GC tissues, non-cancerous gastric mucosa from the GC patients (GC, non-tumor) and normal gastric mucosa from non-GC subjects (Non-GC tissues); C: Impact of *H. pylori* status on the levels of *TGF-β1* promoter methylation in GC tissues (GC, tumor), non-cancerous gastric mucosa from the GC patients (GC, non-tumor) and combined samples (overall). HP+: *H. pylori*-positive; HP-: *H. pylori*-negative; U: Unmethylated; M: Methylated.

**Table 2** Impact of *Helicobacter pylori* on the frequency of transforming growth factor-β1 methylation in gastric tissues

	<i>TGF-β1</i> methylation in GC patients		<i>TGF-β1</i> methylation in Non-GC patients	Total <sup>1</sup>
	Tumor	Non-tumor	Normal gastric mucosa	
<i>H. pylori</i> (+)	21/38 (55.3%)	12/38 (31.6%)	8/22 (36.4%)	41/98 (41.8%)
<i>H. pylori</i> (-)	3/9 (33%)	3/9 (33%)	3/17 (17.6%)	9/35 (25.7%)

<sup>1</sup>Combined tumor and non-tumor tissue. GC: Gastric cancer; TGF-β1: Transforming growth factor-β1; *H. pylori*: *Helicobacter pylori*.

of different concentrations of IL-1β (0.1, 0.25, 1.0 and 2.5 ng/mL) for 12, 24 and 48 h, and *TGF-β1* promoter methylation was then measured by MSPCR. As shown in Figure 2A, IL-1β appeared to induce a dose-dependent methylation of *TGF-β1*, with the strongest methylation being observed in GES-1 cells treated with 2.5 ng/mL of IL-1β for 48 h. Further studies showed that pre-treatment of GES-1 cells with 20 ng/mL of IL-1RA for 1 h could partially abolish the effect of IL-1β on *TGF-β1* methylation (Figure 2B).

***H. pylori* alone dose not induce TGF-β1 methylation in vitro**

Infection of GES-1 cells by *H. pylori* did not induce significant *TGF-β1* promoter methylation (data not shown).

**DISCUSSION**

Promoter hypermethylation leading to epigenetic inactivation of tumor suppressor genes plays a pivotal role in tumorigenesis. Aging, chronic inflammation, and viral and bacterial infections promote methylation of promoter CpG islands and may represent the “environmental” triggers of carcinogenesis. The stomach is one of the organs that constantly undergoes DNA methylation of CpG islands in its epithelial cells. The oncogenic role of *H. pylori* for gastric malignancies, mainly gastric carcinoma and MALT lymphoma, has been well documented, and as such, *H. pylori* has been designated a Class I carcinogen. However, the mechanism by which *H. pylori* induces GC remained poorly defined.

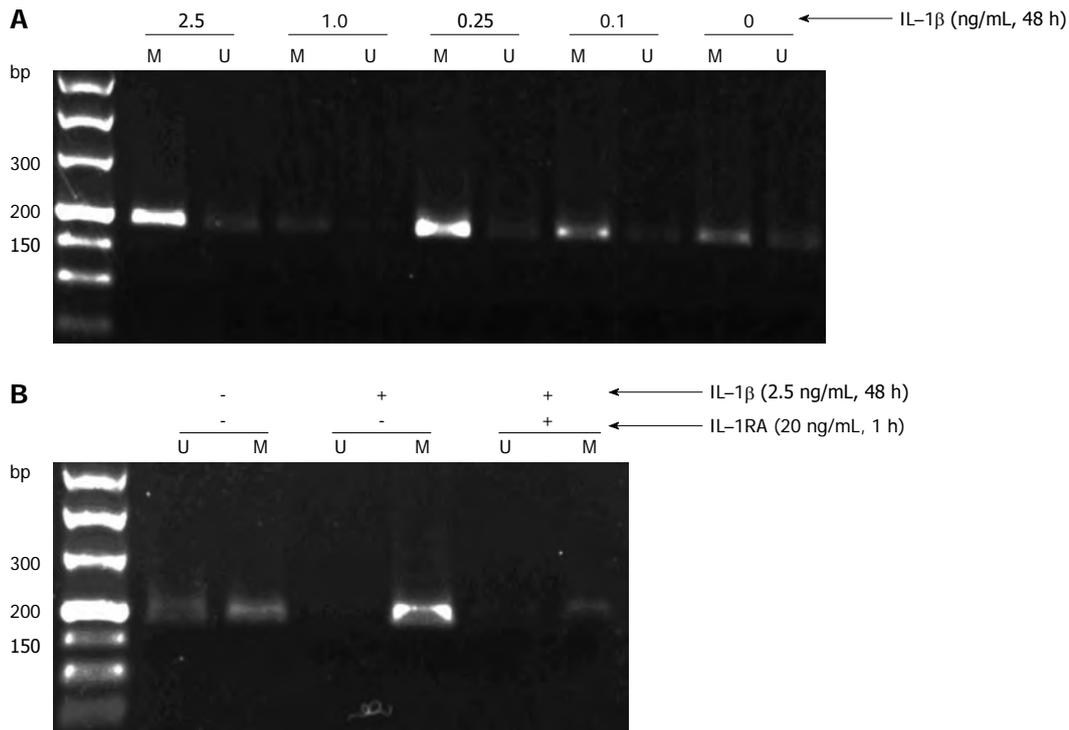


Figure 2 Induction of transforming growth factor- $\beta$ 1 methylation by interleukin-1 $\beta$  in GES-1 cells. A: Treatment of GES-1 cells by interleukin (IL)-1 $\beta$  led to a dose-dependent methylation of transforming growth factor (TGF)- $\beta$ 1; B: IL-1 $\beta$ -induced TGF- $\beta$ 1 methylation in GES-1 cells was partially abolished by IL-1RA. U: Unmethylated; M: Methylated; *H. pylori*: *Helicobacter pylori*.

In the present study, we have revealed, for the first time, that significantly more frequent and higher levels of TGF- $\beta$ 1 promoter methylation are present in GC patients than in non-cancerous controls. Although aging is a recognized risk factor for DNA methylation, from the current study, the impact of age (and sex) on TGF- $\beta$ 1 DNA methylation could be excluded, because our study populations in each group were well-balanced in their distribution of age and sex.

TGF- $\beta$ 1 shows biphasic effects in tumorigenesis<sup>[26]</sup>. In the initial stage, it may function as a tumor suppressor by inhibiting cell growth. This was demonstrated in breast cancer in which constitutive activation of the TGF- $\beta$ 1 pathway prolonged the latency of tumorigenesis or resulted in smaller tumor formation in mice<sup>[27,28]</sup>. However, in the later stage of tumorigenesis (*i.e.*, when the tumors are well established), activation of the TGF- $\beta$ 1 signaling can strongly promote tumor progression<sup>[29]</sup>.

Chronic inflammation-induced methylation of promoter CGIs has been closely linked to the development of human cancers<sup>[30,31]</sup>. In gastric cancer, whether the gene hypermethylation observed in *H. pylori*-infected individuals is caused directly by *H. pylori*, or the gene hypermethylation results from *H. pylori*-induced gastric inflammation remains a topic of debate. In our study, we first examined if *H. pylori* could directly cause methylation of TGF- $\beta$ 1. We made use of a gastric epithelial cell line GES-1, and co-cultured this cell line with *H. pylori*. Contrary to our initial hypothesis, *H. pylori* did not induce apparent promoter methylation of TGF- $\beta$ 1. However, treatment of GES-1 cells with IL-1 $\beta$  led to a marked

methylation of the TGF- $\beta$ 1 promoter, and this was partially reversed by antagonizing IL-1 $\beta$  signaling using its receptor blocker IL-1RA. IL-1 $\beta$  is an important pro-inflammatory cytokine that initiates and amplifies the inflammatory responses to *H. pylori* infection. IL-1 $\beta$  is closely linked to DNA methylation of gastric epithelial cells, particularly in *H. pylori* infected individuals. We think that IL-1 $\beta$  may be an important mediator in *H. pylori*-induced TGF- $\beta$ 1 methylation<sup>[32,33]</sup>.

Our *in vitro* data did not correlate with the *in vivo* data, which showed that *H. pylori* infected individuals, particularly *H. pylori*-positive GC tissues, showed more frequent and higher levels of TGF- $\beta$ 1 methylation. This inconsistency may be explained in several ways. Firstly, the acute infection of GES-1 cells by *H. pylori* may not be a valid model for studying the impact of *H. pylori* on gastric mucosa. It was reported that chronic, rather than acute, *H. pylori* infection was responsible for methylation induction. Secondly, *H. pylori* may not directly induce hypermethylation, but rather it induces inflammation and the production of pro-inflammatory cytokines, such as IL-1 $\beta$ , is likely a contributing factor for TGF- $\beta$ 1 methylation. Our data support this view. Lastly, inflammatory cytokines or molecules may also be involved in *H. pylori*-induced methylation. For example, nitric oxide may be involved in *H. pylori* infection-related DNA methylation<sup>[34]</sup>.

## COMMENTS

### Background

Hypermethylation of promoter CpG islands (CGIs) leads to functional silenc-

ing of some tumor suppressor genes and is thus involved in carcinogenesis. Chronic inflammation is closely associated with cancer formation. Inflammation-induced gene methylation is an important mechanism for inflammation-associated cancers, of which gastric cancer is a classical example. During gastric cancer formation, frequent aberrant CpG methylation has been reported and the role of *Helicobacter pylori* (*H. pylori*) infection during this process is controversial. Inactivation of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) by promoter methylation has been implicated as an important mechanism for the development of several malignancies, such lung and prostate cancers; however, the role of TGF- $\beta$ 1 methylation in gastric cancer remains unknown.

### Research frontiers

Multiple epigenetic and genetic alterations have been identified in gastric cancer (GC) patients. High level of aberrant CpG island methylation in the gastric mucosae is correlated with increased GC risk, and as such, they are increasingly recognized as candidate markers for GC. Clinical and epidemiological studies have demonstrated that *H. pylori* infection is strongly correlated with aberrant methylation in GC. However, the precise molecular mechanism by which *H. pylori* might induce aberrant CpG island methylation remain elusive. GC is a typical inflammation-related malignancy, being closely linked to *H. pylori* induced chronic inflammation in the gastric mucosa. Whether *H. pylori* itself or the chronic inflammation caused by *H. pylori* infection can induce methylation in CpGs remains controversial.

### Innovations and breakthroughs

TGF- $\beta$ 1 is an anti-inflammatory cytokine with multiple effects in many tissues. Many studies have shown that TGF- $\beta$ 1 is overexpressed in epithelial cancers and exerts its transforming potential through several mechanisms, such as stimulating the progression of stromal cells, promoting angiogenesis and suppressing immune surveillance. In the present study, the authors have revealed, for the first time, that significantly more frequent and higher levels of TGF- $\beta$ 1 promoter methylation is present in GC patients with *H. pylori* infection than in non-cancerous controls. *In vitro* studies showed that normal gastric epithelial cell line GES-1 cells exposed to *H. pylori* did not show significant TGF- $\beta$ 1 methylation. However, treatment of the GES-1 cells with IL-1 $\beta$  led to a dose-dependent methylation of TGF- $\beta$ 1. The data show that *H. pylori* may not directly induce hypermethylation, but rather this bacteria induced inflammation and the production of pro-inflammatory cytokines, such IL-1 $\beta$ , is likely a contributing factor for TGF- $\beta$ 1 methylation.

### Applications

High levels of TGF- $\beta$ 1 methylation in the gastric cancer tissue suggest that TGF- $\beta$ 1 functions as a tumor suppressor in *H. pylori* related gastric cancer.

### Terminology

CpG island: CpG islands are DNA segments, at least 0.5 kb in size, that are rich in G:C and CpG content, and are often located in the promoter or 50-exon sequences of genes. Promoter CpG islands have traditionally been thought to be unmethylated in normal cells, with the exception of those on the inactive X chromosome and those associated with imprinted genes. DNA methylation: DNA methylation is the major epigenetic phenomenon of eukaryotic genomes, and involves the addition of a methyl group to the carbon 5 position of the cytosine ring within the CpG dinucleotide. DNA methylation is required for the normal development of cells, whereas aberrant methylation of CpG islands confers a selective growth advantage that results in cancerous growth.

### Peer review

The manuscript by Wang *et al* demonstrates that high levels of TGF- $\beta$ 1 promoter methylation in *H. pylori* positive patients was the result of *H. pylori*-induced inflammation rather than by *H. pylori* itself, and that IL-1 $\beta$  may be an important mediator for *H. pylori*-induced gene methylation during gastric cancer development. The overall goal of the paper is relevant. The data presented are solid and credible. The results are interesting, clinically important and worthy of publication in World Journal of Gastroenterology.

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## Palliative treatment for incurable malignant colorectal obstructions: A meta-analysis

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### Abstract

**AIM:** To perform a meta-analysis of palliative stent placement *vs* palliative surgical decompression for management of incurable malignant colorectal obstructions.

**METHODS:** The databases of Medline, Web of Science, Embase, and the Cochrane Central Register of Controlled Trials were searched from their inception to July 2012 for studies (prospective, retrospective, randomized controlled trials, and case-control trials) designed as comparative analyses of patients with incurable malignant colorectal obstructions treated by self-expanding metallic stents (SEMS) or palliative surgery. No language restrictions were imposed. The main outcome measures were hospital stay, intensive care unit admission, clinical success rate, 30-d mortality, stoma formation, complications, and overall survival time. The data extraction was conducted by two investigators

working independently and using a standardized form. The Mantel-Haenszel  $\chi^2$  method was used to estimate the pooled risk ratios with 95%CI under a fixed-effects model; when statistical heterogeneity existed in the pooled data (as evaluated by  $Q$  test and  $I^2$  statistics, where  $P < 0.10$  and  $I^2 < 25\%$  indicated heterogeneity), a random-effects model was used.

**RESULTS:** Thirteen relevant articles, representing 837 patients (SEMS group,  $n = 404$ ; surgery group,  $n = 433$ ), were selected for analysis. Compared to the surgery group, the SEMS group showed lower clinical success (99.8% *vs* 93.1%,  $P = 0.0009$ ) but shorter durations of hospital stay (18.84 d *vs* 9.55 d,  $P < 0.00001$ ) and time to initiation of chemotherapy (33.36 d *vs* 15.53 d,  $P < 0.00001$ ), and lower rate of stoma formation (54.0% *vs* 12.7%,  $P < 0.00001$ ). Additionally, the SEMS group experienced a significantly lower rate of 30-d mortality (4.2% *vs* 10.5%,  $P = 0.01$ ). Stent-related complications were not uncommon and included perforation (10.1%), migration (9.2%), and occlusion (18.3%). Surgery-related complications were slightly less common and included wound infection (5.0%) and anastomotic leak (4.7%). The rate of total complications was similar between these two groups (SEMS: 34.0% *vs* surgery: 38.1%,  $P = 0.60$ ), but the surgery-related complications occurred earlier than stent-related complications (rate of early complications: 33.7% *vs* 13.7%,  $P = 0.03$ ; rate of late complications: 32.3% *vs* 12.7%,  $P < 0.0001$ ). The overall survival time of SEMS- and surgery-treated patients was not significantly different (7.64 mo *vs* 7.88 mo).

**CONCLUSION:** SEMS is less effective than surgery for palliation of incurable malignant colorectal obstructions, but is associated with a shorter time to chemotherapy and lower 30-d mortality.

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**Key words:** Self-expandable metal stents; Palliative sur-

gery; Incurable malignant colorectal obstruction; Large-bowel obstruction; Treatment outcomes

**Core tip:** This meta-analysis demonstrates the advantages of self-expandable metal stent (SEMS) placement as palliative therapy for incurable malignant colorectal obstructions. Specifically, when compared to the outcomes of surgical treatment, the SEMS treatment is associated with shorter hospital stay and interval to chemotherapy initiation, as well as lower early morbidity and 30-d mortality rates. These advantageous features may surmount the overall lower rate of palliative efficacy when considering treatment options for cases with extensive metastatic disease or severe comorbid medical illness that disqualify a patient from operative candidacy; regardless, SEMS application should be performed as an alternative to surgery with caution.

Zhao XD, Cai BB, Cao RS, Shi RH. Palliative treatment for incurable malignant colorectal obstructions: A meta-analysis. *World J Gastroenterol* 2013; 19(33): 5565-5574 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5565.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5565>

## INTRODUCTION

Malignant colorectal obstruction, a type of large bowel obstruction (LBO), is a frequent and serious complication of advanced cancers, including colorectal cancer or those with near organ (*e.g.*, ovary, vagina, and prostate) or distant metastases<sup>[1]</sup>. LBO initially manifests non-specific gastrointestinal symptoms, such as vomiting, abdominal distention and abdominal pain; however, if left untreated, the condition may progress to a life-threatening status, as the weak necrotic areas of the bowel become more susceptible to tears and a risk for rapid onset infection and septicemia.

The traditional therapeutic approach for LBO is surgical, and the Hartmann's pouch procedure and loop colostomy are the most widely applied surgical methods used for treating obstruction of incurable advanced cancer. Unfortunately, these procedures are associated with substantial drawbacks, including high mortality and morbidity<sup>[2-4]</sup>, as well as detrimental impacts on a patient's quality of life when irreversible ostomies necessitate a colostomy bag<sup>[1,5,6]</sup>. The alternative method of colonic stent insertion was introduced by Dohmoto<sup>[7]</sup> to overcome the risks associated with open surgery. Since then, self-expanding metallic stents (SEMS) have been widely applied to patients with incurable malignant obstructions as palliative treatment or as a bridge to elective primary resection and anastomosis.

SEMS placement is achieved by feeding the metal tube in a collapsed state to the site of obstruction by using a guidewire and visualization by fluoroscopy and/or endoscopy. The inserted stents then undergo passive expansion to create a strong, passable space and relieve

the obstruction. Numerous stents of various lengths and maximal expanded diameter have been designed specifically for treating lower gastrointestinal obstructions, so that the appropriate stent can be chosen for each patient based on location and length of the lesion and severity of the obstruction.

Despite the widespread availability and application of SEMS, its efficacy and safety for treating incurable malignant colorectal obstruction, as compared to that of the traditional surgical approach, has been addressed in relatively few studies with small populations. Thus, this meta-analysis was designed to provide stronger evidence of the outcomes, benefits, and risks of these two palliative treatments through the increased statistical power afforded by pooling data of the previously studied patient populations.

## MATERIALS AND METHODS

### Literature search strategy and data extraction

Two investigators (Zhao XD and Cai BB) performed independent searches of the Medline, Web of Science, Embase, and Cochran Central Register of Controlled Trials databases. These literature collections were queried from inception to July 2012 using the following keywords and medical subject heading terms: stents, colonic stent, colorectal stent, Hartmann's procedures, Hartmann's, colostomy, palliative surgery, intestinal obstruction, large bowel obstruction, colorectal obstruction, comparative study, treatment outcomes, and human. The search strategy was widened or narrowed by applying Boolean operators (NOT, AND, and OR), and no language restriction was applied. All potentially relevant abstracts, studies, and citations were retrieved for review, and the references cited in each were further searched to identify any additional potentially relevant publications.

The two investigators also performed the data extraction (inclusion and exclusion criteria described below), working independently and using pre-determined forms to record first author, year of publication, study design including inclusion and exclusion criteria, and study population characteristics. The extracted datasets were compared and any disagreements were resolved by discussion and consensus.

### Inclusion criteria

Potentially relevant studies were selected for inclusion in the meta-analysis according to the following criteria: (1) comparative analysis of palliative SEMS and palliative surgery for treating malignant colorectal obstructions that were unresectable and had negative margins; (2) patients lacked signs of peritonitis and perforation; (3) reporting of at least one of the outcomes measures listed below; (4) designed as randomized controlled trials (RCTs) or other case-control study; and (5) performed with human patients.

### Exclusion criteria

Studies were excluded from the meta-analysis according to the following criteria: (1) evaluation of SEMS as

**Table 1 Study characteristics of included nonrandomized controlled studies *n* (%)**

Ref.	Design	Diagnosis	Palliative SEMS ( <i>n</i> )	Palliative surgery ( <i>n</i> )	Matching	Female	Study quality (NOS score)
Law <i>et al</i> <sup>[10]</sup>	P	a	30	31	1, 2, 3	21 (34.4)	8
Carne <i>et al</i> <sup>[11]</sup>	R	a	25	19	3	19 (43.2)	4
Johnson <i>et al</i> <sup>[12]</sup>	M	a	20	18	2, 3	17 (47.2)	6
Tomiki <i>et al</i> <sup>[13]</sup>	P	a, b, c	18	17	4	15 (42.9)	4
Ptok <i>et al</i> <sup>[14]</sup>	P	a	40	38	2, 3, 4	34 (44.7)	7
Faragher <i>et al</i> <sup>[15]</sup>	R	a	29	26	1, 2, 4	22 (40.0)	6
Vemulapalli <i>et al</i> <sup>[16]</sup>	R	a	53	70	1, 2, 4	49 (41.2)	5
S�arez <i>et al</i> <sup>[17]</sup>	P	a	45	53	1, 4, 6	31 (31.6)	7
Lee <i>et al</i> <sup>[18]</sup>	P	a	71	73	1, 2, 6	50 (34.7)	7
Lee <i>et al</i> <sup>[19]</sup>	R	a	36	52	1, 2, 4	39 (44.3)	6

Study design is prospective (P), retrospective (R), or case-matched (M); diagnosis is colorectal cancer (a), ovarian cancer (b), or disseminated upper gastrointestinal malignancy (c); matching for age (1), sex (2), diagnosis (3), tumor site (4), or American Society of Anesthesiologists score (6); NOS scores of 5 or more indicate high-quality. SEMS: Self-expanding metallic stents.

**Table 2 Study characteristics of included randomized controlled studies *n* (%)**

Ref.	Design	Diagnosis	Palliative SEMS ( <i>n</i> )	Palliative surgery ( <i>n</i> )	Matching	Female	Study quality (modified Jadad score)
Xinopoulos <i>et al</i> <sup>[20]</sup>	RCT	a,b	15	15	NC	14 (46.7)	High
Fiori <i>et al</i> <sup>[21]</sup>	RCT	a	11	11	1, 2, 4	9 (40.9)	High
van Hooft <i>et al</i> <sup>[22]</sup>	RCT	a	11	10	1, 2, 4, 5	10 (47.6)	High

Study design is randomized controlled trial (RCT); diagnosis is colorectal cancer (a) or ovarian cancer (b); matching for age (1), sex (2), tumor site (4), tumor stage (5), or the publication made no comment (NC) on the matching status; all studies were classified as high-quality according to the modified Jadad score between 4 and 7. SEMS: Self-expanding metallic stents.

a bridge to surgery (SBTS) or as a treatment for benign strictures, or comprehensive studies in which the data could not be clearly separated for exclusion; and (2) missing or unclear data for the outcomes of interest.

**Assessment of methodology quality**

The Newcastle-Ottawa scale<sup>[8]</sup> was employed to assess the quality of non-randomized studies, with scores of ≥ 5 indicating high quality. The modified Jadad score<sup>[9]</sup> was employed to assess the quality of randomized studies, with the cumulative scores of 4 to 7 indicating high quality.

**Statistical analysis**

The meta-analysis was performed by the RevMan 5.0.25 software (The Cochran Collaboration, Oxford, England) and the statistical analysis was carried out by the Stata 12.0 software (StataCorp, College Station, TX, United States). The risk ratios (RRs, with 95%CI) of dichotomous data were estimated by the Mantel-Haenszel  $\chi^2$  method; *P* values of < 0.05 were considered to indicate statistically significant differences between groups. Between-study heterogeneity was evaluated by the *Q* test and *I*<sup>2</sup> statistic, for which *P* values > 0.10 and *I*<sup>2</sup> < 25% indicated a lack of heterogeneity, respectively. In order to broaden the effect estimate in the presence of heterogeneity, the random-effects model was applied for evaluation of the pooled data. Finally, publication bias was estimated by Egger’s and Begg’s funnel plots, for which *P* values > 0.05 indicated a lack of publication bias.

**RESULTS**

**Characteristics of selected studies**

Thirteen studies, including 10 nonrandomized controlled studies<sup>[10-19]</sup> and three RCTs<sup>[20-22]</sup>, met the criteria for inclusion in the meta-analysis. The studies’ characteristics and quality assessment scores are presented in Tables 1 and 2, respectively. Eleven (84.62%) of the studies were categorized as high-quality. The total number of included patients was 837, of which 404 were treated by SEMS (48.3%) and 433 (51.7%) by palliative surgery. Eleven of the studies<sup>[10-12,14-19,21,22]</sup> focused solely on cases with colorectal cancer etiology, and the remaining two studies<sup>[13,20]</sup> also included etiologies of ovarian cancer and disseminated upper gastrointestinal malignancy. The studies also used different definitions of palliative surgery, with four of the studies<sup>[12,13,20,21]</sup> specifically reporting the colostomy procedure and the others reporting primary resection with anastomosis, primary resection without anastomosis, bypass, or Hartmann’s procedure, as well. Complications reported for the total case population were categorized as early (occurring ≤ 30 d post-treatment) or late (occurring > 30 d post-treatment).

**Features of clinical management**

**Length of hospital stay:** The mean length of hospital stay for the pooled SEMS group was significantly lower than that of the pooled surgery group (9.6 d *vs* 18.8 d, *P* < 0.00001).

**Intensive care unit admission:** Three studies<sup>[10,12,18]</sup> reported cases requiring intensive care unit (ICU) admission after treatment. Analysis of the 241 patients, including 119 treated with SEMS and 122 treated with surgery, indicated that the rate of ICU usage was significantly lower in the SEMS group than in the surgery group (0.8% *vs* 18.0%,  $P = 0.001$ ; Figure 1A).

**Time to chemotherapy initiation:** Three studies<sup>[17-19]</sup> reported cases receiving chemotherapy after treatment. Analysis of the 330 patients, including 152 treated with SEMS and 178 treated with surgery, indicated that the mean time to chemotherapy initiation following treatment was significantly lower in the SEMS group than in the surgery group (15.5 d *vs* 33.4 d).

### Short-term outcomes and complications

**Clinical relief of obstructions:** Data of treatment efficacy were available for all cases from all 13 studies. The surgery-treated patients showed a significantly higher rate of clinical relief of obstructions than the SEMS-treated patients (93.1% *vs* 99.8%,  $P = 0.0009$ ; Figure 1B).

**30-d mortality or in-hospital mortality:** Two studies<sup>[19,21]</sup> reported zero mortalities during both the in-hospital stay period and the 30-d follow-up. Meta-analysis of the 688 patients in the remaining ten studies, including 334 treated with SEMS and 354 treated with surgery, indicated that the SEMS group experienced fewer overall deaths than the surgery group (4.2% *vs* 10.5%,  $P = 0.01$ ; Figure 1C).

**Overall, early- and late-onset complications:** Data of treatment-related complications were available for all cases from all 13 studies. Although a slightly lower percentage of the SEMS-treated patients experienced complications, the amount was not significantly different from that in the surgery-treated patients (34.0% *vs* 38.1%,  $P = 0.60$ ; Figure 1D). Nine of the studies<sup>[10,14-18,20-22]</sup> reported data sub-categorized as early complications; while five studies<sup>[15-18,22]</sup> reported data as late complications. Compared to the surgery group ( $n = 326$ ), the SEMS-treated patients ( $n = 300$ ) experienced significantly less early complications (13.7% *vs* 33.7%,  $P = 0.03$ ; Figure 1E) but significantly more late complications (32.3% *vs* 12.7%,  $P < 0.0001$ ; Figure 1F).

### Stent-related complications

Eleven studies<sup>[10-19,22]</sup> reported stent-related complications. The overall rate of perforation was 10.1% (for 367 patients), of stent migration was 9.2% (for 361 patients), of stent obstruction was 18.3% (for 331 patients).

**Surgery-related complications:** Seven studies<sup>[10,11,15-19]</sup> reported surgery-related complications. Six of those studies<sup>[10,15-19]</sup> reported wound infection, and the rate was 5.0% (for 15 patients). Three of those studies<sup>[11,17,19]</sup> reported anastomotic leak, and the rate was 4.7% (for 95 patients).

### Long-term outcomes

**Overall survive time:** Data of survival time were available for all cases from all 13 studies. The overall survival time was similar between the SEMS-treated and surgery-treated patients (7.6 mo *vs* 7.9 mo;  $P > 0.05$ ).

**Stoma formation:** Ten studies<sup>[10-13,15,17-21]</sup> reported stoma formation. Among the 299 patients for whom colonic stent insertion was attempted, 12.7% ( $n = 38$ ) ultimately required a stoma. Among the 315 surgery-treated patients, 54.0% ( $n = 170$ ) required stoma formation. The amount of patients with stoma formation was significantly lower in the SEMS group (*vs* surgery group,  $P < 0.00001$ ; Figure 1G).

### Sensitivity analysis

#### Therapeutic efficacy and outcomes of SEMS and surgery for colorectal cancer-related obstructions:

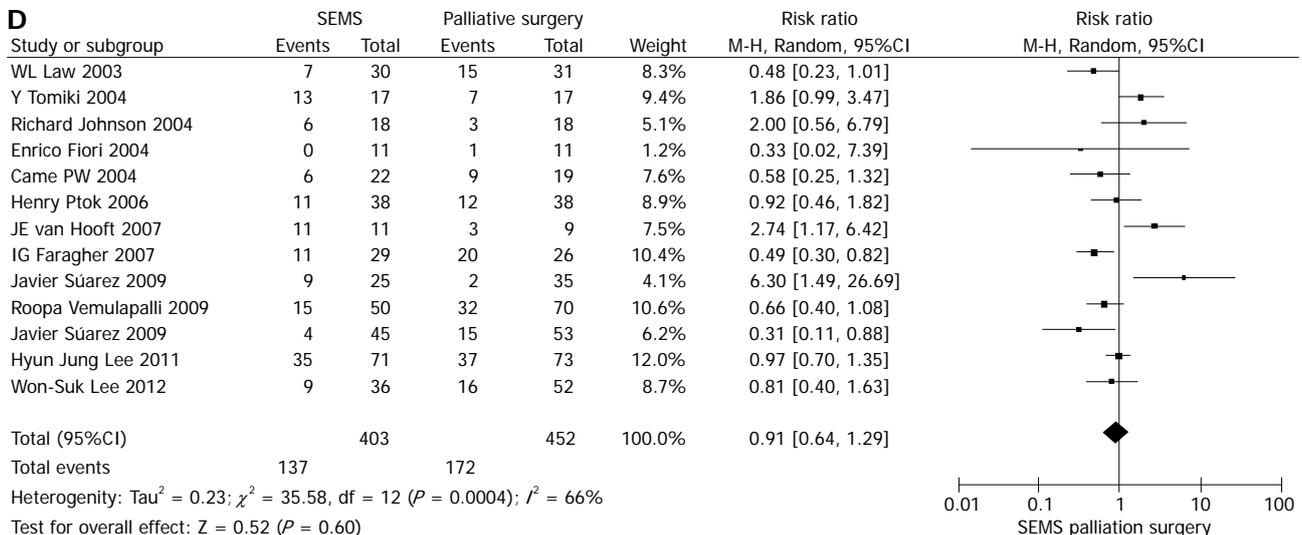
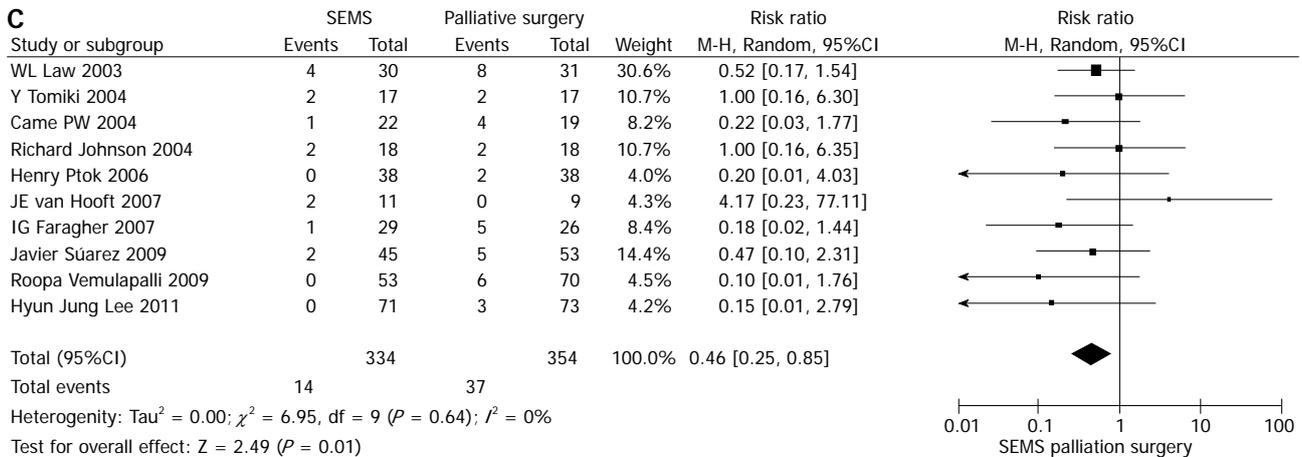
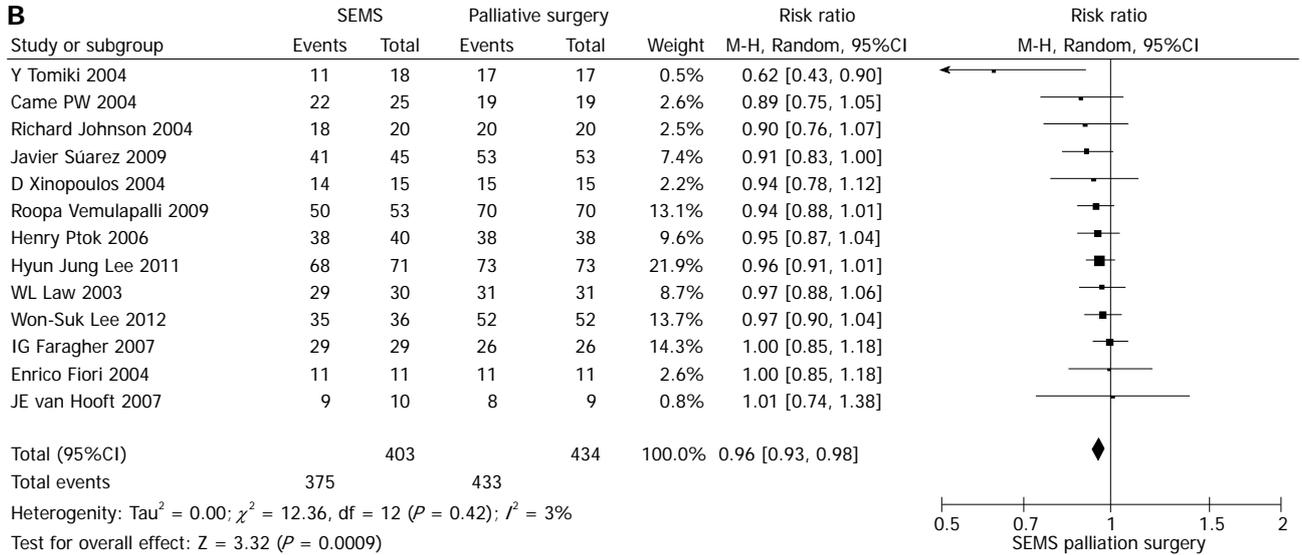
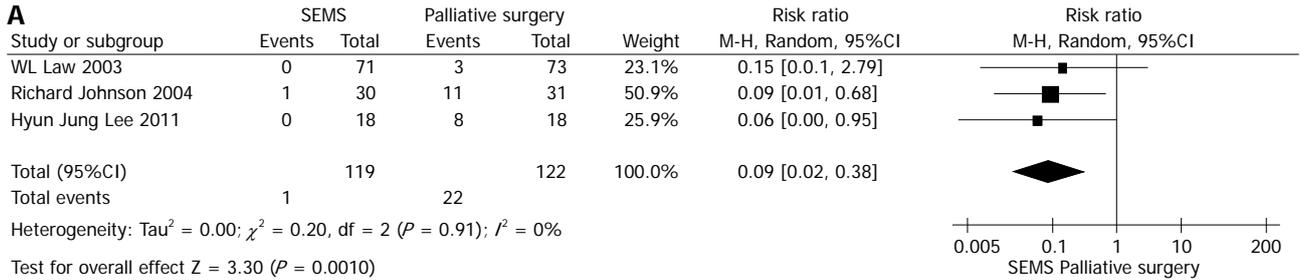
Comparative analysis of the therapeutic efficacies of SEMS and surgery for resolving colorectal cancer-related obstructions<sup>[10-12,14-19,21]</sup> and obstructions caused by other advanced cancers<sup>[13,20]</sup> revealed no differences between the two treatment approaches. However, among the subset of patients with colorectal cancer-related obstructions ( $n = 772$ ), the SEMS-treated patients ( $n = 370$ ) showed significantly lower rates of 30-d mortality (3.79% *vs* surgery-treated patients: 10.4%,  $P = 0.008$ ), early complications (11.2% *vs* 34.7%,  $P = 0.0002$ ), and stoma formation (12.0% *vs* 48.8%,  $P < 0.00001$ ). Unfortunately, these SEMS-treated patients also showed a significantly lower rate of clinical relief of the colorectal cancer-related obstructions (94.6% *vs* 99.8%,  $P = 0.002$ ). No significant difference was observed between the two treatments for total complications (SEMS: 32.1% *vs* surgery: 37.9%,  $P = 0.34$ ) (Table 3).

#### Therapeutic efficacy and outcomes of SEMS *vs* the colostomy surgical treatment:

Four studies<sup>[12,13,20,21]</sup> compared outcomes of SEMS against the colostomy surgical approach. In contrast to the results of SEMS compared to all types of surgeries for treating incurable malignant colorectal obstructions, there was no significant difference found between clinical relief attained by SEMS and colostomy (84.4% *vs* 100%,  $P = 0.18$ ). The SEMS-treated patients, however, did require significantly less stoma formation than the colostomy-treated patients (12.7% *vs* 100%,  $P < 0.00001$ ), and experienced significantly less total complications (23.9% *vs* 41.3%,  $P = 0.04$ ). The rates of 30-d mortality and early complications were not significantly different between the SEMS-treated group and the colostomy-treated group ( $P = 1.00$  and  $P = 0.64$ , respectively) (Table 3).

### Publication bias

As shown in Figure 2, three comparisons showed potential bias: clinical relief of incurable malignant LBO (Egger's test  $P = 0.04$  and Begg's test,  $P = 0.12$ ) and stoma formation (incurable malignant LBO: Egger's test,  $P = 0.001$



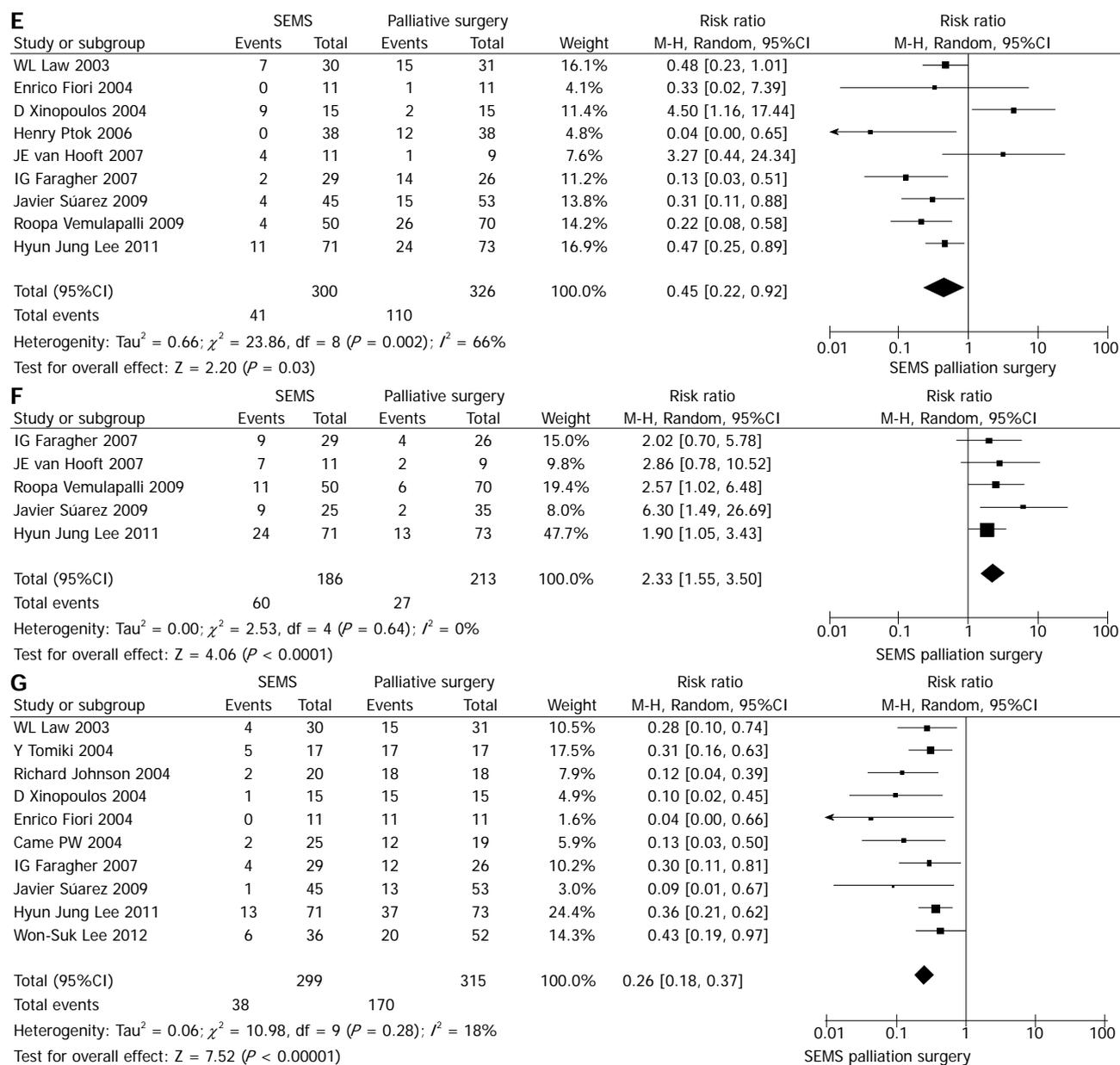


Figure 1 Forest plot. A: Intensive care unit admissions; B: Overall clinical success rates; C: Overall 30-d mortality rates; D: Total complications; E: Complications with early-onset ( $\leq 30$  d post-treatment); F: Complications with late-onset ( $> 30$  d post-treatment); G: Stoma formation. SEMS: Self-expanding metallic stents.

and Begg's test,  $P = 0.03$ ; incurable malignant colorectal cancer-related obstructions: Egger's test,  $P = 0.005$  and Begg's test,  $P = 0.04$ ). However, the statistical analysis revealed no evidence of publication bias among any of these comparisons.

## DISCUSSION

Previous studies have demonstrated the risks associated with the traditional surgical approach for treating malignant LBO, namely high rates of morbidity, mortality, and stoma formation<sup>[23,24]</sup>. The less invasive alternative approach of colonic stent insertion, particularly of SEMS, promised to overcome the high hospitalization costs and poor quality of life related to these outcomes. While subsequent meta-analyses have been conducted to investigate

the benefit and risk of endoscopic SBTS<sup>[25-28]</sup>, no study to date had performed a focused comparison of palliative SEMS and palliative surgery for treating incurable malignant LBO-as is described herein.

In the current meta-analysis, palliative surgery was found to be superior to SEMS for decompressing incurable malignant LBO; while this finding is contrary to the majority of individual studies of this subject<sup>[29,32]</sup>, it is consistent with the investigations by Cirocchi *et al.*<sup>[28]</sup> and Sagar<sup>[33]</sup>. An important distinguishing feature among these collective studies is the variable definitions of palliative surgery that were used as the basis of analysis; in addition, these studies have yet to address whether and to what extent primary tumor resection affects the mean survival time of those patients suffering from advanced cancer<sup>[34,35]</sup>. In our meta-analysis of eleven studies, the

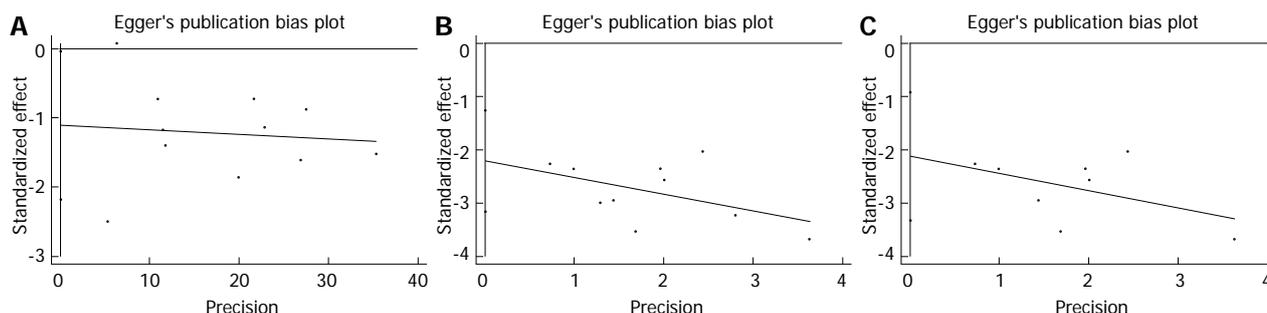


Figure 2 Egger's and Begg's plots of publication bias among the included trials. A: Clinical relief of incurable malignant large bowel obstruction (LBO); B: Stoma formation of incurable malignant LBO; C: Stoma formation of incurable colorectal cancer obstruction.

**Table 3 Sensitivity analysis of the included studies**

	Studies (n)	Patients (n)	RR (95%CI)	P value
Studies including colorectal cancer only				
Clinical success rate	11	772	0.96 (0.93, 0.98)	0.002
30-d mortality	9	654	0.42 (0.22, 0.80)	0.008
Total complications	12	821	0.84 (0.59, 1.20)	0.340
Early complications	8	596	0.35 (0.20, 0.60)	0.0002
Stoma formation	8	550	0.26 (0.17, 0.39)	< 0.00001
Studies including colostomy only				
Clinical success rate	4	127	0.89 (0.76, 1.05)	0.18
30-d mortality	2	70	1.00 (0.27, 3.68)	1.00
Total complications	3	92	1.79 (1.03, 3.09)	0.04
Early complications	2	52	1.80 (0.16, 20.79)	0.64
Stoma formation	4	124	0.16 (0.07, 0.38)	< 0.00001

overall clinical success rate of SEMS treatment ranged from 70%-95%. A previous multicenter study<sup>[36]</sup> of SEMS with long-term follow-up revealed that the clinical success rate increased gradually over time (87.8% at 30 d, 89.7% at 3 mo, 92.8% at 6 mo, and 96% at 12 mo). The follow-up period in our included studies are different but all within 12 mo and the clinical success rate was approximately similar. In addition, our meta-analysis revealed that obstructions caused by colorectal cancer benefited more from the surgical approach. Fernández-Esparrach *et al.*<sup>[37]</sup> have reported a similar finding and hypothesized that the severe complications associated with the SEMS procedure, such as migration, obstruction and perforation, limited its long-term clinical efficacy. Moreover, the authors advised that adjunct palliative chemotherapy may help to promote the life expectancy of SEMS-treated patients. A retrospective study conducted in Korean patients advanced gastric cancer<sup>[38]</sup> also indicated that SEMS insertion was less effective than emergency surgery for the palliative treatment for colorectal obstructions. In light of these previous findings, and in agreement with the opinions expressed by other interested groups in this field<sup>[39,40]</sup>, it is possible that the clinical stent success rate observed in our current meta-analysis had nothing to do with the stent placement or the etiology of the obstructions. Indeed, Sebastian *et al.*<sup>[31]</sup> suggested that the clinical success rate of stenting is mainly associated with the site and extent of the obstruction.

Our meta-analysis also indicated that SEMS treatment is associated with shorter lengths of hospital stay,

reduced ICU admissions, fewer stoma formation, and shorter time to initiation of adjunct chemotherapy; These findings are consistent with results from other relevant studies<sup>[30,32,33,41]</sup> and suggest that the less trauma endured produced by the SEMS approach eliminates delay of post-procedure chemotherapy, thereby promoting beneficial patient outcome. It was unfortunate that the current meta-analysis was limited by a lack of comparative data concerning quality of life outcome and cost-effectiveness between these two palliative treatments; analysis of such data will be necessary for comprehensively assessing the feasibility of these palliative management approaches for advanced disease. Only one of the studies included in the meta-analysis, a RCT<sup>[20]</sup> comprised of 30 patients, attempted to address the monetary expense of stent placement, as compared to colostomy treatment; however, the analysis was abandoned due to the high rate of colonic perforation that occurred in the nonsurgical arm. However, some studies<sup>[32,42]</sup> that did not meet the criteria for inclusion in our meta-analysis have suggested that SEMS may be less costly than the conventional surgical approach for treating colonic cancer obstructions; but, we cannot comment on the quality or appropriateness of these data or the implications related to our findings.

The safety of stent placement was also evaluated in the current meta-analysis. Although SEMS insertion is considered a less invasive method than surgery, and advanced procedure-related devices, such as hydrophilic elastic guidewires and stent delivery systems, have improved the ease and successful application of this method, complications still occur. Fortunately, the majority of complications are minor, such as low fever and abdominal discomfort, and resolved easily by medication. While less frequent, the major complications of the stent procedure, such as bleeding, colonic perforation, stent migration and stent occlusion, can be life-threatening<sup>[43]</sup>. In a systematic review<sup>[30]</sup> of 88 articles reporting on stent-related complications in cases of LBO, the median rates of stent migration, perforation, and reconstruction were reported as 11%, 4.5% and 12%, respectively. In the current meta-analysis, the rates of perforation and reconstruction were slightly higher; we believe this finding reflects the fact that data on perforations caused by tumor infiltration were included in the analysis and that the data on reconstructions included not only the etiologies of tu-

mor ingrowth/overgrowth and stent migration, but also of fecal implant.

The contributing factors to complications of stent insertion have been extensively studied. Factors related to stent type have been particularly well studied, and it is believed that covered stents provide the optimal resistance to tumor ingrowth, thereby helping to reduce reconstruction events, while uncovered stents are believed to minimize stent migration<sup>[30,39,44]</sup>. The type of stent, however, does not appear to be related to perforation events<sup>[30]</sup>, nor to have a significant effect on the safety of stent placement<sup>[45]</sup>. Furthermore, a retrospective analysis of uncovered SEMS for treating primary colorectal cancer *vs* non-colorectal extrinsic cancer found no significant difference in migration or occlusion events<sup>[46]</sup>. That study also suggested that insufficient stent expansion (< 70%) at 48 h after insertion may be a predictor of subsequent stent occlusion. Another retrospective analysis of 168 SEMS-treated LBO patients<sup>[47]</sup> identified five risk factors of therapeutic inefficacy, including male sex, complete obstruction, stent diameter  $\leq$  22 cm, premature dilation of the stent, and operators' experience. In addition, subsequent chemotherapy, especially Bevacizumab therapy, was demonstrated to nearly triple the risk of perforation. This latter finding was not supported by the study by Kim *et al*<sup>[39]</sup>, who demonstrated that chemotherapy had no affect on migration or reconstruction and that stent length had no relationship with complications, but showed that stent diameter < 24 cm had negative impact on palliative SEMS migration. In another study, stent migration was shown to occur more frequently in the distal colon<sup>[51]</sup>.

Despite significant improvements in the surgical procedures used for managing incurable malignant colorectal obstructions, the perioperative morbidity and mortality rates have remained high. Similarly, the patients treated with surgery in the current meta-analysis experienced appreciable levels of anastomotic dehiscence, wound infection, and death. The former two complications may have a negative influence on tumor recurrence, metastasis, and long-term survival. In the current meta-analysis, a greater number of surgery-treated patients died within 30 d after treatment, as compared to those treated with SEMS. While this result is contrary to those obtained with other similar patient series<sup>[30,33]</sup> and meta-analyses comparing SBTS<sup>[25-28]</sup>, it may be explained by the lower amount of total complications that were experienced by the overall SEMS-treated group. Another study also found significantly lower complications in a stent-treated group, but we cannot comment on the related implications for our findings as the previous data had significant heterogeneity<sup>[25]</sup>. In an attempt to address this issue, we performed sub-group analysis of the complications, independently assessing the early- and late-onset complications; the results indicated that surgery had a higher risk of early complications, while SEMS insertion had a higher risk of late complications. Future studies should further investigate the roles of early and late complications in therapeutic efficacy and overall survival.

Two of the studies<sup>[13,20]</sup> included in the overall meta-analysis were excluded from the focused comparison of SEMS and surgery outcomes for incurable colorectal-related obstructions. The results were not impacted by their removal and were in accordance with the findings reported by Kim *et al*<sup>[40]</sup>. Then, we investigated the comparison between SEMS and colostomy for incurable malignant LBO (using four studies). Unlike the previous results, these results suggested that, compared to colostomy, SEMS could be an effective palliative treatment for incurable malignant LBO; no significant difference was found for the clinical success rates between groups with fewer stoma, but the 30-d mortality and the complications should be taken into account. Unfortunately, the current meta-analysis was underpowered to investigate the differences in overall survival time between these two groups.

Other limitations of our meta-analysis design may have impacted our results and their interpretation. First, only three of the 13 included studies are RCTs. Second, the pooled sample size was still relatively small and the data from the included studies was not uniform for the outcome measures. Third, publication bias existed among four of the studies; indeed, a general limitation of all meta-analyses is publication bias introduced by the fact that positive results are more likely to be published. To overcome these limitations, long-term RCTs should be conducted with large numbers of patients to achieve a sufficient level of statistical power for accurately estimating the optimal palliative treatment for incurable malignant LBO.

In summary, palliative SEMS does not appear to have a significant advantage over palliative surgery for decompressing incurable malignant colorectal obstructions, regardless of etiology; however, the use of colonic stents is safe. The shorter interval to chemotherapy and significantly lower rates of 30-d mortality and short-term complications suggest that SEMS may be a reasonable alternative for treating patients with extensive metastatic disease or who are poor operative candidates due to severe comorbid medical illnesses.

## COMMENTS

### Background

Malignant colorectal obstruction is a common and serious complication of advanced cancer. The traditional treatment approach, surgery, is associated with high risks of morbidity and mortality. The more recently developed approach of stent insertion is less invasive and has been widely applied, especially using self-expanding metallic stents (SEMS), but its risks and benefits in patients with incurable malignant large bowel obstruction (LBO) remain to be definitively established.

### Research frontiers

The current meta-analysis was carried out to comparatively assess the outcomes of palliative surgery and palliative SEMS insertion in patients with incurable malignant LBO; the main outcome measure included length of hospital stay, intensive care unit admission, clinical success rate, 30-d mortality, complications, stoma formation, and overall survival time.

### Innovations and breakthroughs

The current meta-analysis demonstrated the advantages of SEMS as a pal-

liative therapy for incurable malignant LBO, in terms of shortened durations of hospital stay and time to chemotherapy initiation, and decreased rates of 30-d mortality and early-onset complications. However, SEMS failed to show a greater efficacy than palliative surgery for resolving obstructions.

### Applications

The results from this meta-analysis suggests that colonic stent insertion may be a safe and feasible alternative palliative treatment for patients who are otherwise poor candidates for the traditional surgical treatment, such as those with extensive metastatic disease or severe comorbid medical illnesses. SEMS is not absolutely recommended, however, since it is associated with significant late-onset complications and mortality. Until further randomized controlled trials, with large patient populations, are carried out, application should be considered on a case-by-case basis.

### Terminology

SEMS: expandable metal tubes that are placed in the collapsed state at the site of obstruction by means of a guidewire and fluoroscopy and/or endoscopy visualization; gradual, automatic expansion to the maximum diameter of the stent serves to relieve the obstruction and create a strong and passable space. Meta-analysis: the collection, combination, and analysis of data from multiple previously completed studies on a particular topic of interest that is carried out with the aim of increasing statistical power to draw stronger conclusions about a controversial subject.

### Peer review

The current meta-analysis was designed to evaluate the risks and benefits of SEMS treatment for incurable malignant colorectal obstructions, as compared to surgical treatment. The analysis included a total of 13 studies, nine of which scored high upon established quality assessment systems. The research design is solid, and its results have clinical relevancy as they demonstrate that, in patients with incurable malignant colorectal obstruction, stent placement improves treatment outcome, specifically by shortening the time to chemotherapy initiation and lowering the 30-d mortality rate.

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## Lactic acidosis during telbivudine treatment for HBV: A case report and literature review

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### Abstract

All oral nucleoside analogues against hepatitis B virus, with an exception of telbivudine, have been reported causing lactic acidosis (LA). Here we report the first case of chronic hepatitis B developing severe refractory LA during telbivudine monotherapy. A 36-year-old man of Chinese origin received telbivudine antiviral treatment for chronic hepatitis B. After 11 mo of therapy, he developed anorexia, nausea, and vomiting with mild muscle weakness. The patient was found with elevated serum creatine phosphokinase up to 3683 U/L (upper limit of normal 170 U/L) and marked LA. LA did not resolve immediately following discontinuation of telbivudine. His condition began to improve after he-

modialysis treatment for 16 times and usage of glucocorticosteroid. The patient fully recovered after 16 wk of treatment. This is the first documented case with severe LA caused by telbivudine monotherapy. Besides serum creatine phosphokinase, blood lactate level should also be closely monitored in patients receiving telbivudine.

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**Key words:** Lactic acidosis/hyperlactatemia; Telbivudine; Hepatitis B virus; Nucleoside analogue; Adverse effects

**Core tip:** Myopathy is the most common side effect resulting from telbivudine. Lactic acidosis (LA) is rare but fatal, and LA caused by telbivudine has never been reported. Here, we report the first case of chronic hepatitis B developing severe refractory LA during telbivudine monotherapy. This case shows that telbivudine may cause muscle damage and even lead to fatal LA in chronic hepatitis B patients. Patients under telbivudine treatment should be closely monitored for muscular, blood lactate and other mitochondrial toxicity associated side effects.

Jin JL, Hu P, Lu JH, Luo SS, Huang XY, Weng XH, Zhang JM. Lactic acidosis during telbivudine treatment for HBV: A case report and literature review. *World J Gastroenterol* 2013; 19(33): 5575-5580 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5575.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5575>

### INTRODUCTION

Suppression of hepatitis B virus (HBV) DNA is a principal goal in treating chronic hepatitis B because this was shown to significantly improve liver histology as well as

to decrease rates of hepatic complications and hepatocellular carcinoma. Current treatment options are pegylated interferon alpha and nucleoside analogues including lamivudine, telbivudine, entecavir, adefovir dipivoxil and tenofovir disoproxil. These agents have relatively fewer side effects than interferon alpha, and generally well tolerated<sup>[1]</sup>. Infrequent but serious adverse events have been reported in clinical trials and post-marketing surveillance in individual cases. Lactic acidosis (LA) is one of the severe adverse events and has been reported in the patients treated by all the other four nucleoside analogues except for telbivudine.

All of the five approved oral antiviral agents for HBV treatment can inhibit the polymerase activity of HBV, leading to a reduction in viral replication and serum HBV DNA levels. At the same time, some of these agents have a low level of activity against the human mitochondrial DNA (mtDNA) polymerase gamma and can lead to impaired mitochondrial replication with mitochondrial loss or dysfunction<sup>[1]</sup>. Clinical manifestations of mitochondrial toxicity vary based on the affected tissues, but may include myopathy, neuropathy, hepatic steatosis, pancreatitis, macrocytosis, nephrotoxicity, hyperlactatemia and LA. All nucleoside analogues have a “black box” warning regarding potential mitochondrial toxicity in their product labeling.

Telbivudine is a potent oral nucleoside analogue approved for the treatment of chronic hepatitis B in 2006 at a dose of 600 mg/d. A significantly higher incidence of grade 3-4 serum creatine phosphokinase (CPK) elevation (*i.e.*, > 7 times upper limit of normal) was reported in a large, multinational registration clinical trial<sup>[2]</sup>. However, to date, there has been no published report of LA caused by telbivudine monotherapy. Here, we report a case of LA during telbivudine treatment, discuss the pathophysiology, clinical features and potential treatment of LA.

## CASE REPORT

The patient is a 36-year-old, HIV-negative young male farmer. He was admitted to our hospital because of nausea and vomiting repeatedly for 40 d.

He had suffered from chronic hepatitis B for 13 years. His liver function test (LFT) revealed an intermittent elevation of alanine aminotransferase (ALT) levels between 1999 and 2011, and recovered to normal level after some symptomatic treatment. In September 2011, his LFT became abnormal again, the ALT was 704 U/L and HBV DNA was  $7.0 \times 10^7$  copies/mL, HBV markers showed HBsAg, HBeAg and HBcAb were positive. Subsequently, he began to take telbivudine 600 mg/d regularly (Figure 1). In early September 2012 (47 d before admission), he began to develop anorexia, nausea and vomiting without apparent causes. There were no other concurrent symptoms, such as fever, headache, abdominal pain and altered level of consciousness. But he had mild muscle pain and weakness. The diagnostic workup including gastroscopie, cranial CT and abdominal plain

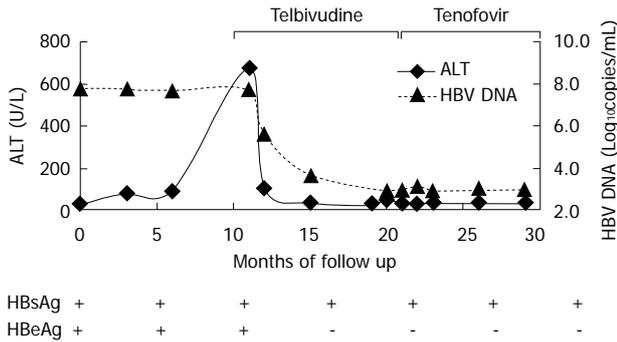
film revealed bilateral multiple renal calculi. CPK was significantly elevated at 3683 U/L (normal range: 25-170 U/L) 20 d before admission (Figure 2). The arterial blood gas analysis at that time showed pH 7.41, carbon dioxide partial pressure 37.2 mmHg, oxygen partial pressure 87.1 mmHg, actual bicarbonate 23.2 mmol/L, standard bicarbonate 23.6 mmol/L, base excess -1.4 mmol/L, and blood lactate level 4.4 mmol/L (upper limit of normal 2.5 mmol/L). It was considered that hyperlactatemia was caused by telbivudine at a local clinic. Subsequently telbivudine was discontinued.

However, the patient's condition continued to deteriorate despite alkalization treatment. Two weeks before admission, his CPK level decreased to 1183 U/L, but the arterial blood gas analysis demonstrated a worsening of metabolic acidosis: pH 7.2, actual bicarbonate 10.6 mmol/L, base excess 15.8 mmol/L, and blood lactate level elevated to 10.7 mmol/L (Figure 3). The clinical symptoms included persisting nausea and vomiting. The blood lactate level rose further to more than 12 mmol/L (the upper limit can be detected in the laboratory) (Figure 3). A week before admission, the patient received eight times of hemodialysis treatment at a local clinic. His blood lactate returned to a normal level each time after hemodialysis, however, it would rebound the next day. The patient was eventually transferred to our hospital because of refractory LA. On the day of admission, the blood lactate was 7.93 mmol/L, ALT was 42 U/L, aspartate aminotransferase was 66 U/L, LDH was 349 U/L and CPK was 632 U/L. Physical examination on admission revealed waddling gait and proximal muscular weakness in both lower limbs, quantitative value was 4 grade.

The patient was noticed to have a history of hypokalemic periodic paralysis for more than 10 years after a serious inquiry. His first attack was the most severe one, with paralysis affecting both of his legs but recovered after potassium supplement. There was no further event in the recent years.

The examination after admission also revealed hypothyroidism: TSH 12.39 mIU/L, T4 110.1 nmol/L, T3 1.31 nmol/L, and FT4 14.42 pmol/L. B-mode ultrasonography showed diffuse enlargement of thyroid. Endocrinologist consultation considered a subclinical hypothyroidism, and 25 µg euthyrox was prescribed daily.

Electromyography revealed mild myopathic changes. Prolonged exercise test was normal. Muscle biopsy on left biceps revealed moderate variation in fiber size as well as increased muscle nucleus (Figure 4). A substantial number of degenerative muscle fibers occurred. Regeneration of muscle fiber could be seen, with no inflammatory cells infiltration. Mitochondrial damage was identified by modified Gomori trichrome stain and other histopathological studies. Modified Gomori trichrome staining revealed many ragged red fibers (RRF); reduced form of nicotinamide-adenine dinucleotide (NADH) and succinic dehydrogenase (SDH) staining showed disorganized enzyme activity in the fibers with RRF. ATP staining showed mosaic arrangement of type I and type



**Figure 1** Progression of serum hepatitis B virus DNA and aminotransferase. Telbivudine was introduced when alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA level was both high. The indication was clear and sufficient, and lactic acidosis happened after 11 mo of antiviral treatment when liver function was controlled well. HBV DNA continued to be normal after telbivudine was stopped and changed to tenofovir soon after.

II fibers. Oil Red O staining showed that several muscle fibers were filled with increased lipid droplets. Histo-Immunochemical tests were Rod-Dystrophin (+), C-Dystrophin (+), N-Dystrophin (+), Dysferlin (+), Merosin (+),  $\alpha$ -Sarcoglycan (+),  $\beta$ -Sarcoglycan (+), and  $\gamma$ -Sarcoglycan (+). The patient was diagnosed with LA (type B2), HBeAg negative chronic hepatitis B and drug-induced myopathy.

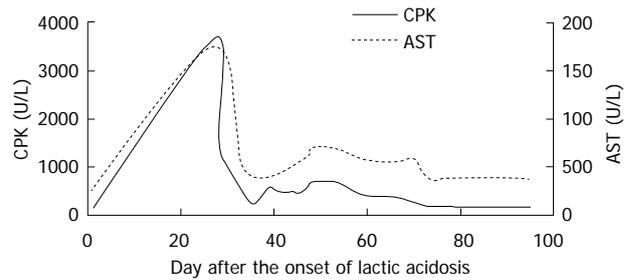
He was given hemodialysis for more than eight times after admission. The blood lactate level reduced to normal range (less than 2.5 mmol/L) after hemodialysis but slightly elevated the following day. The symptoms of nausea and vomiting completely recovered, so the hemodialysis was discontinued. He was given hydration, alkalization and supplementation with Coenzyme Q 10 and Levocarnitine. Two weeks after hemodialysis, the blood lactate level still fluctuated between 5 and 7 mmol/L. As a result, methylprednisolone tablets (24 mg/d) was given. Meanwhile, HBV DNA was rechecked and showed a slight rebound at  $1.59 \times 10^3$  copies/mL, consequently tenofovir (300 mg/d) was given to suppress the HBV.

In the following two weeks, his blood lactate level returned to a normal range, and the HBV DNA was undetectable (less than 1000 copies/mL), so methylprednisolone was tapered off within a ten-week period. The patient has remained very well and followed up regularly to date.

## DISCUSSION

Our patient had marked LA without evidence of infection or organ hypoperfusion. It is very likely that his acidosis was secondary to the nucleoside analogue, telbivudine, during treatment of HBV.

In basic terms, lactic acid is the normal endpoint of the anaerobic breakdown of glucose in the tissues. In the setting of decreased tissue oxygenation, lactic acid is produced as the anaerobic cycle is utilized for energy production. The normal blood lactate concentration in unstressed patients is 0.5-1 mmol/L. Lactate concentration



**Figure 2** Progression of serum creatine kinase level. Creatine kinase (CPK) elevated at the very beginning of lactic acidosis and returned to normal range quickly. AST: Aspartate aminotransferase.

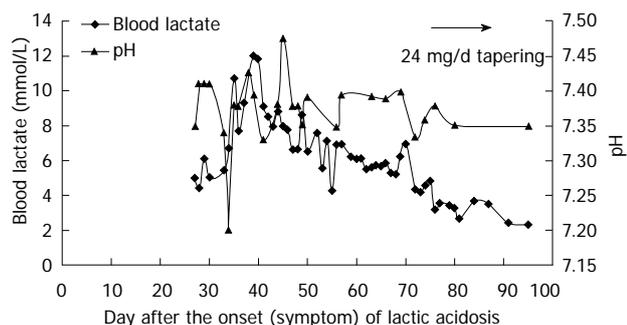
of less than 2 mmol/L can be considered to be normal in patients with critical illness. Hyperlactatemia is defined as a persistent, mild to moderate (2-4 mmol/L) increase in blood lactate concentration without metabolic acidosis; whereas LA is characterized by constant increased in blood lactate levels (usually > 5 mmol/L) in association with metabolic acidosis (usually present as pH < 7.3 and serum bicarbonate < 10 mmol/L)<sup>[1]</sup>.

The LA syndrome linked to nucleoside analogue is associated with steatosis, abnormal mitochondrial appearance and function, pancreatitis, neuropathy, and myopathy. The onset may be abrupt or insidious, it generally begins with nausea, vomiting, and abdominal pain. It will progress to tachypnea, shortness of breath, and hypoxia. Patients with severe LA may subsequently develop renal failure, liver failure, coagulopathy, seizures, arrhythmias, and even death. The patient reported here was a severe LA case with a lactate level of more than 12 mmol/L and pH value of 7.2. His blood lactate level did not recover to normal even after hemodialysis treatment for 16 times.

The Food and Drug Administration approved oral nucleoside analogues for HBV treatment, including lamivudine, adefovir, telbivudine, entecavir, and tenofovir, are well tolerated. However, these still carry the “black box” warning for the potential development of mitochondrial damage with resultant LA based on the data from the human immunodeficiency virus (HIV) treatment literature<sup>[3-7]</sup> and the experience using fialuridine (FIAU) in HBV treatment<sup>[8]</sup>.

Lamivudine<sup>[4,5]</sup> and tenofovir<sup>[3,7]</sup> associated LA was reported only in HIV patients treated with combination regimens (Table 1), while their mitochondrial toxicity is far less than those antiretroviral nucleoside analogues. The risk of LA with entecavir treatment in chronic hepatitis B patients remain controversial. However, it was reported to occur more often in patients with impaired liver function<sup>[1,9,10]</sup>, especially in those with high MELD (model for end stage liver diseases) scores and multi-organ failure (Table 1). Report of LA caused by adefovir is rare, and all reported cases were present in a combination regimens<sup>[9]</sup>.

Telbivudine, as with all the other approved nucleoside analogues, has a potential of mitochondrial toxicity which will lead to LA in theory. However, no single case has been reported to date. This will be the first documented case of type B LA in a chronic hepatitis B patient who



**Figure 3** A refractory lactic acidosis case and the fluctuation of blood lactate level. Symptoms lasted more than 3 mo and recovered slowly after 16 times of hemodialysis and small dosage of glucocorticoid helped to resolve the persistent serum lactate elevation.

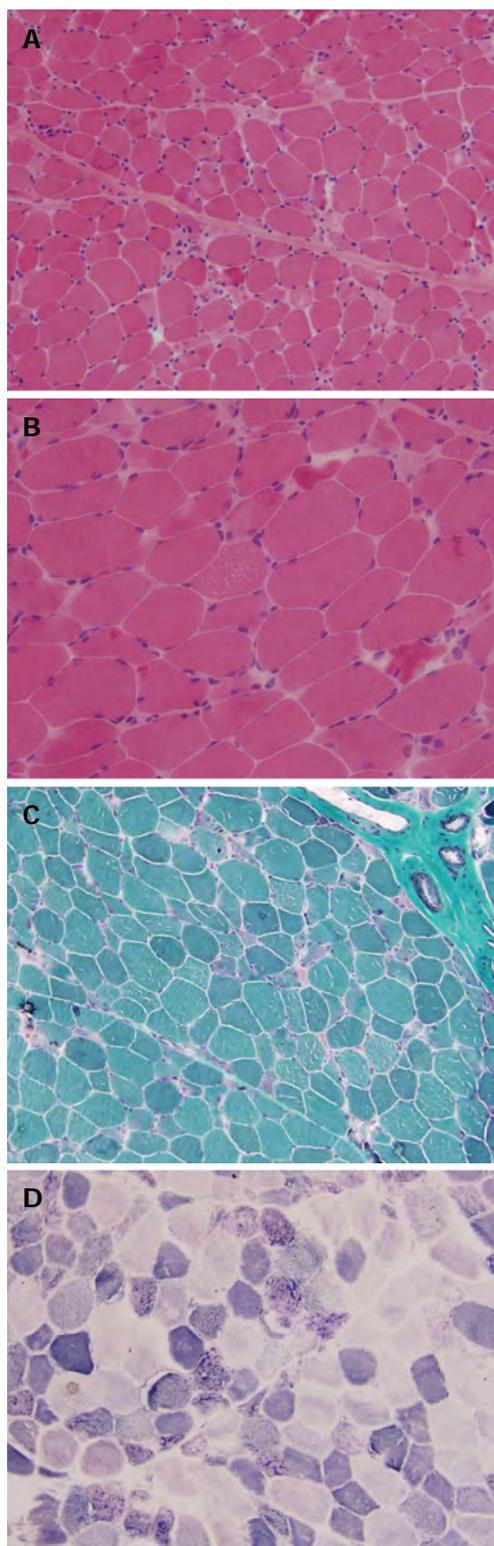
received telbivudine monotherapy.

Among the five nucleoside analogues approved for the use in hepatitis B, the inhibitory strength of mtDNA polymerase gamma in an *in vitro* test system is actually far less than that seen in antiretroviral agents. In the registration trial of telbivudine for HBV, the side-effect profile of telbivudine was generally favorable<sup>[2]</sup> and similar to comparator arm of lamivudine throughout 2 years of treatment. There was no LA case reported, however, a significantly higher incidence of grade 3 to 4 serum CPK elevations (*i.e.*, 7 times upper limit of normal) was noted in telbivudine-treated compared to lamivudine-treated patients at 2 years (12.9% *vs* 4.1%).

We noticed that our patient had a history of hypokalemic periodic paralysis. Hypokalemic periodic paralysis is an autosomal-dominant disorder characterized by episodic attacks of muscle weakness with hypokalemia. Whether there was pre-existence of myopathy in our patient prior to telbivudine treatment is uncertain, only transient CPK elevation was observed and most of time the CPK value was normal before LA occurred. The reason that LA and CPK elevation does not co-exist in most cases during monotherapy of nucleoside analogues in chronic hepatitis B patients is unclear. Interestingly, our case is a rare incident where CPK elevation and LA occurred simultaneously (Table 1). This case has suggested that besides CPK, serum lactate level should also be monitored closely during the treatment of telbivudine.

LA can be divided into 2 categories, type A and type B. Type A is LA occurring in association with clinical evidence of poor tissue perfusion or oxygenation of blood (*e.g.*, hypotension, cyanosis, cool and mottled extremities). Type B is LA occurring when no clinical evidence of poor tissue perfusion or oxygenation exists. Type B can be divided into 3 subtypes based on underlying etiology. Type B1 occurs in relation to systemic disease, such as renal and hepatic failure, diabetes and malignancy. Type B3 is due to inborn errors of metabolism. Type B2 is caused by several classes of drugs and toxins, including biguanides, alcohols, iron, isoniazid, zidovudine, and salicylates.

Our patient had marked LA without evidence of in-



**Figure 4** Histopathology of muscle biopsy specimens showed mitochondrial toxicity. A: Many regenerating and necrotic muscle fibers, mild nuclear proliferation and necrosis around muscle fibers (HE, magnification × 200); B: Part of muscle fibers filled with fatty droplets (HE, magnification × 400); C: Ragged red fibers under envelope of shrinking muscle cells (modified Gomori trichrome stain, magnification × 200); D: The figure revealed the structural disorders of mitochondria. The myocytes different in size; Type I and Type II muscle fibers showed mosaic arrangement (nicotinamide-adenine dinucleotid, magnification × 200).

**Table 1** Characteristics of patients with lactic acidosis treated with nucleoside analogues

Patient ID	Age (yr)	Liver condition	Underlying disease	Child-Pugh	MELD score	Drug	LA therapy	Peak lactate (mmol/L)	Nadir pH	BE (mmol/L)	Peak CPK (U/L)	Prognosis	Ref.
1	35	CHB	HOKPP	A	7	LDT	11 mo	> 12	7.2	-15.8	3683	Resolved	This paper
2	36	OLT, ITBL	-	C	38	ETV	9 mo	5.20	7.2	-18	Normal	Resolved	[7]
3	79	ALF	-	-	29	ETV	6 d	20.82	7.1	-17	Normal	Death	[7]
4	60	OLT, re-cirrhosis	-	C	28	ETV	1 mo	3.86	7.4	-5	Normal	Resolved	[7]
5	60	Cirrhosis HCC	-	B	25	ETV	10 d	6.77	7.3	-12	Normal	Resolved	[7]
6	61	Cirrhosis HCC	-	B	22	ETV	4 d	2.70	7.4	-6	Normal	Resolved	[7]
7	63	CHB, HCC	massive bilobar pneumonia	C	30	ETV	10 d	9.20	7.24	-	Normal	Resolved	[8]
8	54	CHB, cirrhosis	CML	C	24	ETV + ADV	10 d	9.50	6.95	-	Normal	Resolved	[9]
9	42	HIV	-	A	7	HARRT (stavudine + LAM)	9 mo	5.48	7.15	-	Normal	Resolved	[6]
10	51	HIV	DM	A	7	HARRT (tenofovir)	12 mo	6.40	7.21	-	Normal	Resolved	[7]

MELD: Model for end stage liver diseases; LA: Lactic acidosis; BE: Base excess; CPK: Creatine phosphokinase; CHB: Chronic hepatitis B; OLT: Orthotopic liver transplantation; ITBL: Ischemic-type biliary lesions; ALF: Acute liver failure; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; HOKPP: Hypokalemia periodic paralysis; CML: Chronic myelogenous leukemia; DM: Diabetes mellitus; LAM: Lamivudine; ETV: Entecavir; ADV: Adefovir; LDT: Telbivudine; HARRT: Highly active antiretroviral treatment; Lactate mmol/L  $\times$  9.608 = mg/dL.

fection or organ hypoperfusion. In view of the fact that no other underlying causes were identified, his acidosis may be due to telbivudine (Type B2 LA). The patient also had mild muscle pain and proximal muscle weakness consistent with a myopathy, as shown on the electromyography. It is likely LA and myopathy arise from the same pathological origin, *i.e.*, mitochondrial dysfunction. Indeed, subsequent muscle biopsy showed RRF, lipid storage and mitochondrial dysfunction, which indicated the mitochondrial toxicity.

Management options for type B LA may include treatment for primary diseases, renal replacement therapy, bicarbonate alkalization and supplementation with thiamine, L-acetylcarnitine as well as Coenzyme Q 10<sup>[10]</sup>. In term of nucleoside analogues, discontinuation should be instantaneously. Most of the LA cases can resolve rapidly after discontinuation of the causative drug. Majority of the patients who developed LA secondary to nucleoside analogues had a good outcome. The recovery progression for our patient was slow with a total period of more than three months. The symptoms improved after hemodialysis therapy for 16 times, and blood lactate level normalized to the upper limit of normal, but halted for a period of time. No plausible reasons can be found for this phenomenon, but small dosage of glucocorticoid seems to be effective. The use of low-dose glucocorticoid for a short period of time may have an unusual effect. However, a larger controlled clinical trial is required for further clarification. It should be applied cautiously by an experienced clinical hepatologist.

This case shows that telbivudine may cause muscle damage and even lead to fatal LA in telbivudine-treated chronic hepatitis B patients. Thus patients receiving tel-

bivudine should be closely monitored for muscular abnormalities, blood lactate level and other mitochondrial toxicity associated side effects.

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**E-Editor** Ma S



## Selective endoscopic ligation for treatment of upper gastrointestinal protuberant lesions

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### Abstract

This study explored the clinical value of endoscopic ligation for the treatment of upper gastrointestinal (GI) protuberant lesions in children. According to the appearance and size of lesions, we used different ligation techniques for the treatment of the lesions. Endoscopic ultrasonography was used for preliminary characterization of the lesions. One case diagnosed with Peutz-Jeghers syndrome was successfully treated by a detachable snare. Two cases with semi-pedunculated or broad-base lesions originating from the submucosal layer of the upper GI were treated with endoscopic variceal ligation; endoscopic examination showed that one case had complete healing 11 wk after ligation, while an ulcer scar was observed at the ligation site after 6 wk in the other case. All lesions were successfully ligated at the first attempt. No significant complications occurred either during or after the procedure. Selective endoscopic ligation of upper GI lesions is an effective and safe treatment for upper GI protuberant lesions in

children.

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**Key words:** Endoscopy; Ligation; Endoscopic ultrasonography; Protuberant lesion; Children

**Core tip:** Endoscopic ligation is an effective method in the management of protuberant lesions. It is less invasive and less expensive than surgical interventions. However, there are few studies of this technique in the treatment of upper gastrointestinal (GI) lesions in children. This paper reports selective endoscopic ligation for the treatment of different upper GI protuberant lesions in children. Endoscopic ultrasonography was used to determine the depth of invasion and provided a preliminary characterization of the lesions.

Wang L, Chen SY, Huang Y, Wu J, Leung YK. Selective endoscopic ligation for treatment of upper gastrointestinal protuberant lesions. *World J Gastroenterol* 2013; 19(33): 5581-5585 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5581.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5581>

### INTRODUCTION

Protuberant lesions in the gastrointestinal (GI) tract may cause clinical symptoms (e.g., abdominal pain, bleeding, intussusception, obstruction) and have malignant potential. In addition, the presence of the lesion is a source of psychological distress. With the development of endoscopic techniques and devices, endoscopic treatment has become an effective method for protuberant lesions in the gastrointestinal tract. It is less invasive than surgical interventions.

Endoscopic ligation has been widely used in the management of post polypectomy bleeding, bleeding of esophageal and gastric varices, and angiodysplasias<sup>[1]</sup>. In

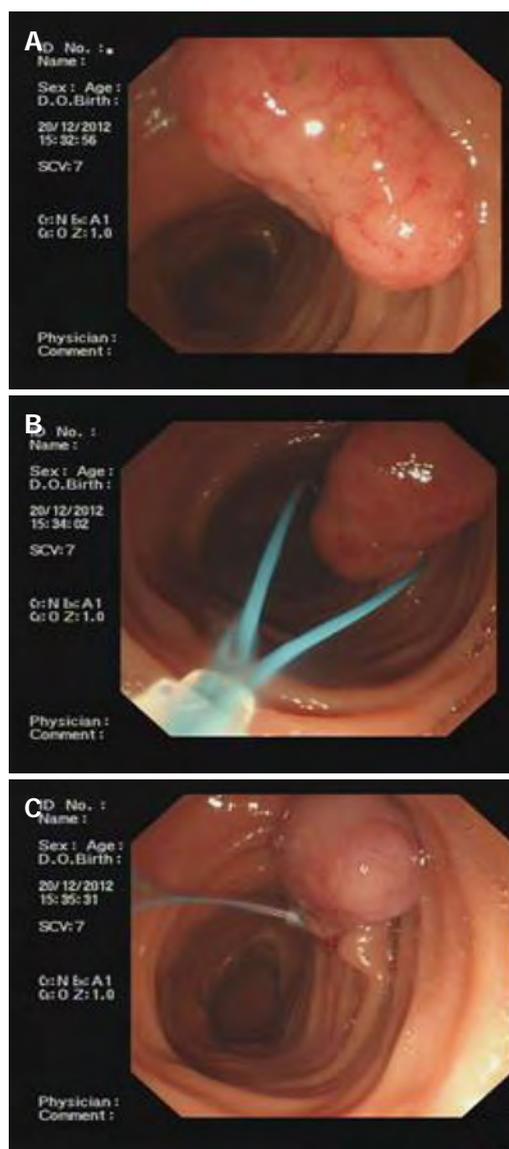


Figure 1 Imaging in case 1. A: Endoscopic view of the large pedunculated polyp in the descending part of duodenum; B: Endoscopic ligation with a detachable snare; C: Endoscopic view of the ligated polyp, with the endoloop placed around the stalk.

2004, Sun *et al.*<sup>[2]</sup> first reported endoscopic band ligation without electrosurgery was an effective and safe treatment for resection of small upper GI leiomyoma. They found most leiomyomas could slough spontaneously within 3.6 to 4.5 wk. Complications related to use of electrosurgery were avoided. There was a case report about colonoscopic polypectomy with a detachable snare to remove a large juvenile polyp in 1-year-old girl<sup>[3]</sup>. However, there are few published reports regarding endoscopic ligation to treat upper GI protuberant lesions in children. Here, we report three patients with upper GI protuberant lesions who received endoscopic treatment with ligation.

## CASE REPORT

### Case 1

A 4-year-old girl presented with abdominal pain for 6 mo.

She was diagnosed with Peutz-Jeghers syndrome. Under general anesthesia, conventional upper GI endoscopic examination (GIF-XQ260, Olympus, Japan) revealed multiple polyps. A large, 25 mm × 15 mm, pedunculated polyp with a hyperemic and edematous surface was found in the descending part of the duodenum (Figure 1). Another polyp with a thick stalk, 16 mm × 10 mm in size, also appeared in the opposite side of the duodenal papilla. To remove the two large polyps safely, we performed endoscopic ligation with detachable snares (MAJ339, Olympus, Japan). The device was composed of an elliptically shaped nylon loop and a silicone-rubber stopper which maintained the tightness of the loop. The nylon loop was placed at the base of the stalk, tightened around the stalk, then the stopper was detached from the device. We observed the color change in the target lesion to ensure proper tightening, when the ligated lesion changed to dark red because of congestion. A smaller lesion, 3 mm × 3 mm, was found in the gastric body. It was removed using the electrocoagulation technique. No complications occurred during or after the procedure using the detachable snare.

### Case 2

A 10-year-old boy with nausea and belching for 2 mo underwent upper gastrointestinal endoscopy (GIF-XQ260, Olympus, Japan) in our hospital. The result revealed a protuberant lesion located in the gastric antrum. Endoscopic ultrasonography (EUS) was performed using a radial echoendoscope with a 20 MHz catheter probe (UM-DP12-25R, Olympus, Japan), and showed a 6.5 mm × 5.0 mm hypoechoic, homogeneous lesion originating from the submucosal layer. The lesion did not involve the muscularis propria. Endoscopic ligation was performed using an attached endoscopic variceal ligation (EVL) device (Six Shooter Saeed Multiband Ligator, Wilson-Cook Medical, Winston-Salem, NC, United States), which aspirated the lesion into the ligator cap, and then an elastic rubber band was released then tightened around the base of the lesion (Figure 2). The goal of ligation was to create a polypoid form with a pseudo stalk. For complete ligation, suction should be maintained for at least 1 min before releasing the rubber band. There were no significant procedure-related complications. The lesion completely healed within 11 wk after the ligation.

### Case 3

A 10-year-old boy with an episode of recurrent abdominal pain was referred to our hospital for gastrointestinal endoscopy (GIF-XQ260, Olympus, Japan). Under general anesthesia, EUS examination showed a hypoechoic homogeneous mass, 13.6 mm × 9.2 mm in size, originating from the submucosal layer of the duodenal bulb (Figure 3). It did not extend to the muscularis propria. The ligation of this lesion was carried out with the EVL device. The lesion was sucked sufficiently into the ligator cap and the band was tightened to ligate the base of the tissue. No complications were reported during the proce-

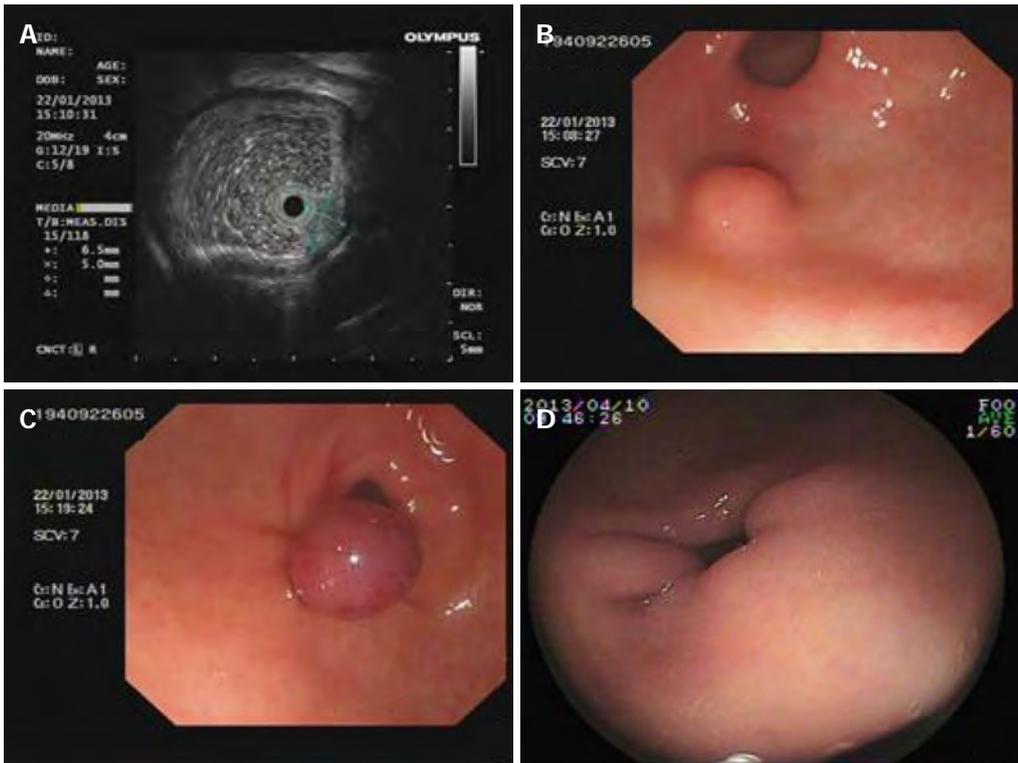


Figure 2 Imaging in case 2. A: Endoscopic ultrasonography image of the submucosal lesion; B: Endoscopic view of the lesion in gastric antrum; C: Endoscopic view showing the ligated lesion; D: Endoscopic image at 11 wk after ligation, showing healing at the ligation site.

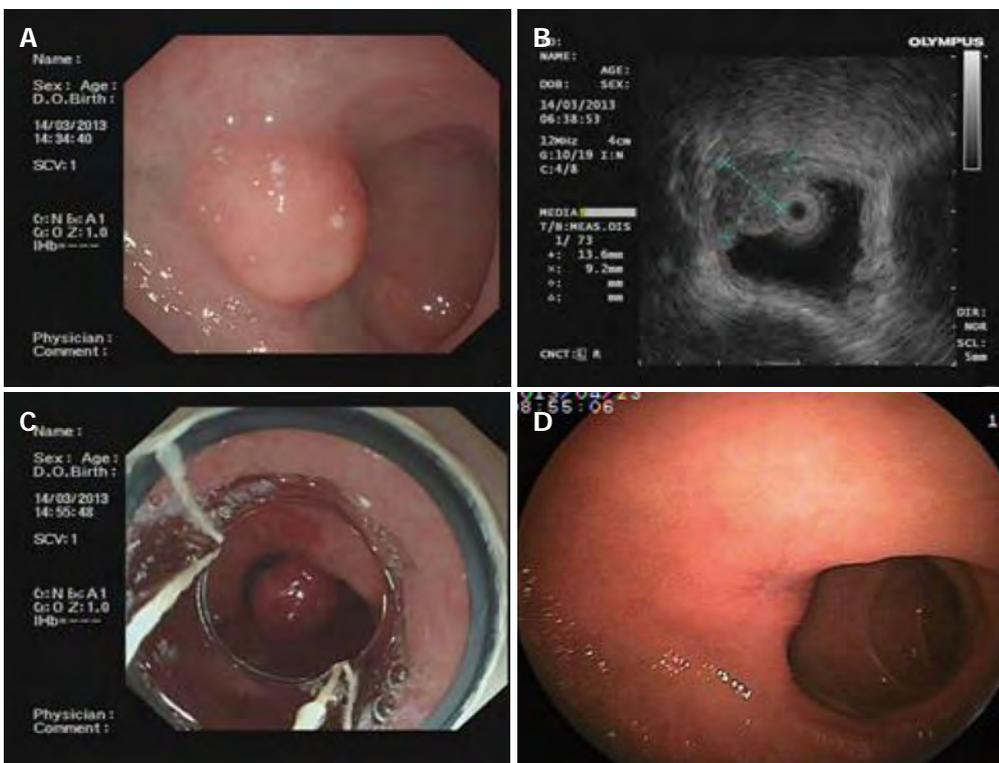


Figure 3 Imaging in case 3. A: Endoscopic view of the protuberant lesion in the duodenal bulb; B: Endoscopic ultrasonography image of the size of the lesion; C: Endoscopic view of the ligated lesion using the endoscopic variceal ligation device; D: Endoscopic view of an ulcer scar at the ligation site after 6 wk.

ture. Four days later, GI endoscopy showed sloughing of the raised lesion, and an ulcer could be observed. A scar

was seen at the ligation site on a follow-up examination of 6 wk later.

## DISCUSSION

There has been remarkable progress in the use of endoscopic treatment for gastrointestinal diseases. A detachable snare for endoscopic use was first developed by Hachisu<sup>[4]</sup>. Large polyps or other raised lesions have been successfully removed by detachable snares. In a randomized trial, Iishi *et al.*<sup>[5]</sup> used endoscopic ligation with a detachable snare for the stalk of a large pedunculated polyp and evaluated the safety and effectiveness of the procedure in comparison with conventional endoscopic snare polypectomy. Results showed that no bleeding occurred in 47 patients assigned to colonoscopic polypectomy with a detachable snare, but bleeding occurred in five of 42 patients who received conventional colonoscopic polypectomy. Moreover, the use of a detachable snare reduced the duration of hospitalization after polypectomy. In 2005, Raju *et al.*<sup>[6]</sup> first described a new technique for successful removal of a large pedunculated, 4 cm wide, broad-based colonic lipoma using endoloops without the need for cautery. Lee *et al.*<sup>[7]</sup> reported that nine cases diagnosed with large pedunculated GI submucosal tumors were successfully treated by endoloop ligation in 2008, and the tumors were removed within 4 wk. Recently, a trial was published to evaluate the clinical impact of selective ligation using a detachable snare for small intestinal polyps in three adult patients with Peutz-Jeghers syndrome<sup>[8]</sup>. The technique of endoscopic ligation is safer than conventional snare polypectomy or endoscopic mucosal resection. It could reduce the risk of bleeding and injury of the deeper tissue layers. However, to our knowledge, the effect of endoscopic ligation for upper GI protuberant lesions in children has not been reported.

According to our experience, there are two aspects to be considered in endoscopic treatment. One is the appearance of elevated lesions. A study described some instances where the use of a detachable snare was ineffective for colonoscopic polypectomy of large polyps with thin stalks or for lesions that were semi-pedunculated<sup>[9]</sup>. It is difficult to tighten the lesion sufficiently in semi-pedunculated or broad-based lesions, as the loop is more likely to slip off. A target lesion positioned at the 5 o'clock to 7 o'clock position is easier to remove by an endoloop<sup>[1]</sup>. Huang *et al.*<sup>[10]</sup> reported the methodology for different lesions using an EVL device or endoloop. Small GI stromal tumors ( $\leq 12$  mm) were treated by endoscopic band ligation with an EVL device. Large pedunculated tumors ( $> 12$  mm) could be managed by endoscopic ligation with a detachable endoloop, while ligation of large sessile tumors was carried out with a large-sized transparent cap plus an endoloop. In our cases, we used a detachable snare for large pedunculated lesions. If the lesion was semi-pedunculated or sessile, endoscopic ligation was carried out with an attached band ligator device. In one of the two cases using an EVL device, the lesion was larger than 12 mm in diameter. For smaller lesions (3 mm  $\times$  3 mm), conventional electrocoagulation was performed. Thus, selective ligation is essential to avoid complications.

The second consideration is that endoscopic band li-

gation is associated with a risk of perforation. It is necessary to be careful to avoid aspirating excessive tissue into the cap. Perforations were reported in two studies after endoscopic band ligation<sup>[11,12]</sup>; two cases were GI stromal tumors in the gastric fundus, and one was a gastric submucosal tumor partly connecting with the muscularis propria. The reason might be that all the layers of the gastric wall were ligated. It seems that the risk of perforation is greater with deeper layer tumors. Determination of the origin of the lesion and appropriate suction force are essential factors that should be considered.

Endoscopic ligation allows the lesion to slough spontaneously. The main limitation of this technique is the difficulty in retrieving the tissue specimen. Histopathologic diagnoses could not be made. However, protuberant lesions in children are mostly considered as benign and to have very low potential for malignant transformation. Sun *et al.*<sup>[13,14]</sup> reported that EUS-assisted band ligation with systematic follow-up by EUS was an effective treatment for small upper GI stromal tumors. EUS was used to determine the histologic layer of origin and evaluate whether the mass was confined completely by the band. It has significant value in the diagnosis of submucosal lesions. In the present study, EUS examination was used to identify the depth of invasion. It provided preliminary identification of the property of the lesion. We recommend that our patients should have close follow-up in order to detect any recurrence early.

In conclusion, endoscopic ligation appears to be a feasible method for removal of upper GI lesions in children. According to the characteristics and volume of the lesions, selecting the correct application of ligation and controlling the suction force can reduce the risk of related complications, such as hemorrhage and perforation. In addition, combining endoscopic ligation with EUS examination may be a useful technique for submucosal tumors in children. Pediatric experience with this endoscopic technique remains limited. Thus, studies involving more subjects and longer follow-up are needed to further define the clinical role of endoscopic ligation in children.

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## Gallstone ileus: Case report and literature review

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Author contributions: Dai XZ and Li GQ contributed equally to this work; Dai XZ and Zhang CY designed the report; Dai XZ and Li GQ were attending doctors for the patients; Dai XZ, Li GQ and Zhang CY performed the surgical operation; Zhang F and Wang XH performed imaging diagnosis; Zhang CY organized the report; Dai XZ wrote the paper.

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### Abstract

Gallstone ileus (GI) is characterized by occlusion of the intestinal lumen as a result of one or more gallstones. GI is a rare complication of gallstones that occurs in 1%-4% of all cases of bowel obstruction. The mortality associated with GI ranges between 12% and 27%. Classical findings on plain abdominal radiography include: (1) pneumobilia; (2) intestinal obstruction; (3) an aberrantly located gallstone; and (4) change of location of a previously observed stone. The optimal management of acute GI is controversial and can be: (1) enterotomy with stone extraction alone; (2) enterotomy, stone extraction, cholecystectomy and fistula closure; (3) bowel resection alone; and (4) bowel resection with fistula closure. We describe a case to highlight some of the pertinent issues involved in GI management, and propose a scheme to minimize recurrent disease and postoperative complications. We conclude that GI is a rare condition affecting mainly the older

population with a female predominance. The advent of computed tomography and magnetic resonance imaging has made it easier to diagnose GI. Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications.

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**Key words:** Gallstone ileus; Fistula closure; Intestinal obstruction; Bowel obstruction; Enterolithotomy

**Core tip:** We present the case of a 56-year-old female who presented at our institution with symptoms of bowel obstruction. Abdominal computed tomography (CT) and exploratory laparotomy revealed a large gallstone in the terminal ileus. She underwent enterolithotomy and had an uneventful postoperative course. The literature suggests that gallstone ileus (GI) is a rare condition affecting mainly the older population and has a female predominance. CT and magnetic resonance imaging have made it easier to diagnose GI. Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications.

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### INTRODUCTION

Gallstone ileus (GI) is characterized by occlusion of the intestinal lumen as a result of one or more gallstones<sup>[1,2]</sup>. According to reports from the 1990s, GI is a rare complication of gallstones that occurs in 1%-4% of all cases of bowel obstruction and in  $\leq 25\%$  of cases of non-strangulated small-bowel obstruction in patients aged  $> 65$  years. The mortality associated with GI ranges between



Figure 1 Abdominal radiographs were normal.

12% and 27%<sup>[3]</sup>. GI accounts for only 0.095% of cases of mechanical bowel obstruction in the United States<sup>[4]</sup>. The optimal management of acute GI is controversial<sup>[3,4]</sup>. We describe a case here to highlight some of the pertinent issues involved in GI management, and propose a scheme to minimize recurrent disease and postoperative complications.

## CASE REPORT

A 56-year-old female presented with intermittent vomiting and abdominal pain for 7 d. This patient had cholecystolithiasis for 10 years and had been treated with antibiotics. She had been treated in another hospital with intravenous fluids and antibiotics. She had undergone gastrointestinal decompression and regulation of water and electrolytes for approximately 1 wk, but the symptoms had not abated.

One week after the latest attack, she was referred to our hospital for nausea, vomiting, constipation and abdominal pain. Upon examination, her abdomen was moderately distended, and tympanic and high-pitched bowel sounds were audible. Rectal examination was normal. Blood tests revealed an elevated total leukocyte count ( $14.4 \times 10^9$  cells/L) and unremarkable liver function test values. Plain radiographs of the abdomen were normal (Figure 1). She was treated with intravenous fluids and antibiotics. However, 2 d after hospital admission, clinical deterioration was investigated with computed tomography (CT). CT demonstrated a small-bowel obstruction due to a 50-mm calculus within an ileal loop. CT showed air in the gallbladder and adhesions between the thickened gallbladder wall and duodenal wall. A severe air-fluid level was also seen (Figure 2). We therefore made a diagnosis of GI.

The patient underwent an exploratory laparotomy, during which a wide fistula from the gallbladder to the second part of the duodenum was found. Abdominal adhesions around the gallbladder were very severe. Exploration revealed massively dilated loops of the small bowel proximal to the distal ileum. An obstruction was seen 100 cm from the terminal ileum, where an enterotomy was made to reveal a large gallstone (5 cm  $\times$  3 cm  $\times$  4 cm).

The gallstone was removed and the enterotomy repaired in two layers (Figure 3). The patient had an uneventful postoperative course and was discharged home on postoperative day 10.

## DISCUSSION

GI is more common in women, and the ratio of females to males is 3.5 to 1<sup>[4]</sup>. The gallstone may enter the intestine through a fistula and it can impact anywhere in the gastrointestinal tract<sup>[5]</sup>. The gallstone must be  $\geq 2$ -2.5 cm in diameter to cause obstruction<sup>[6-9]</sup>. As shown by Reisner and Cohen, impaction of the stone can occur in any part of the bowel, *i.e.*, the ileum (60.5% of cases), jejunum (16.1%), stomach (14.2%), colon (4.1%), and duodenum (3.5%). It can also be passed spontaneously (1.3%)<sup>[3,10]</sup>. It occurs most frequently in the terminal ileum and the ileocecal valve because of their narrow lumen and potentially less active peristalsis<sup>[10]</sup>.

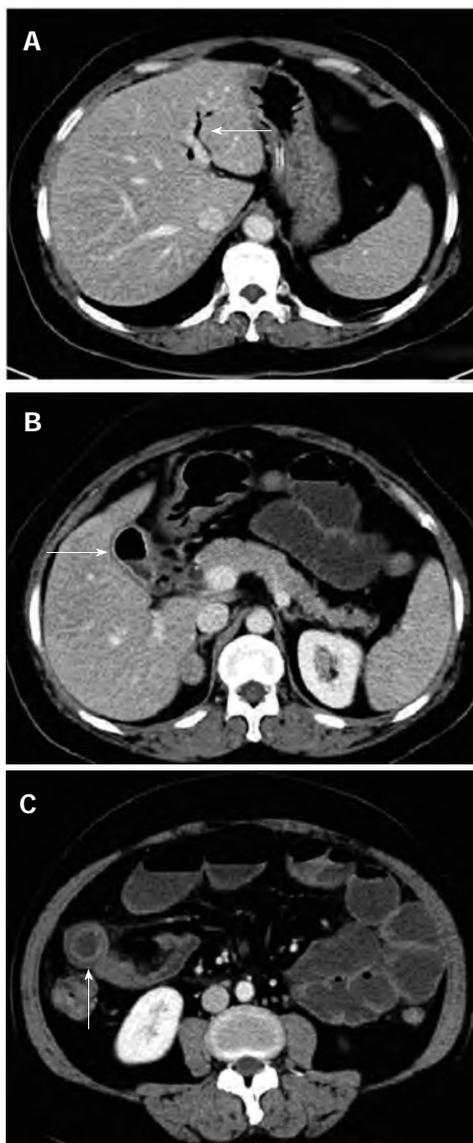
If GI occurs in elderly patients with comorbidities, the often vague, intermittent symptoms may delay the diagnosis by days<sup>[3]</sup>. Presentation is typically non-specific, and often with intermittent symptoms of nausea, vomiting, abdominal distension and pain. We should pay more attention to those patients who have the history of cholecystolithiasis and with symptoms such as nausea, vomiting, abdominal distension and pain. In the past, confirming the diagnosis was difficult, but the advent of CT and magnetic resonance imaging (MRI) has made it easier to diagnose GI<sup>[10,11]</sup>.

Classical findings on plain abdominal radiography include: (1) pneumobilia; (2) intestinal obstruction; (3) an aberrantly located gallstone; and (4) a change in location of a previously observed stone<sup>[9,11-14]</sup>. The widespread use of CT with an overall sensitivity, specificity, and diagnostic accuracy of 93%, 100% and 99%, respectively, has aided diagnosis<sup>[14]</sup>. Interestingly, no aerobilia which can be easily detected by transabdominal ultrasound may be one reason for the delayed diagnosis. Additionally, the absence of significant calcification of the stone reduces the chance of an early diagnosis. In 50% of cases, the diagnosis is often only made at laparotomy<sup>[3]</sup>.

GI is a mechanical intestinal obstruction caused by impaction of gallstones within the lumen of the bowel. Most reports indicate that stones smaller than 2.5 cm usually pass through spontaneously, so conservative treatment (decompression by nasogastric drainage) is conducted before a decision is made to remove the impacted stone by surgical means<sup>[6,8]</sup>.

Management of GI is controversial and includes: (1) enterotomy with stone extraction alone; (2) enterotomy, stone extraction, cholecystectomy and fistula closure; (3) bowel resection alone; and (4) bowel resection with fistula closure<sup>[4,6,15]</sup>.

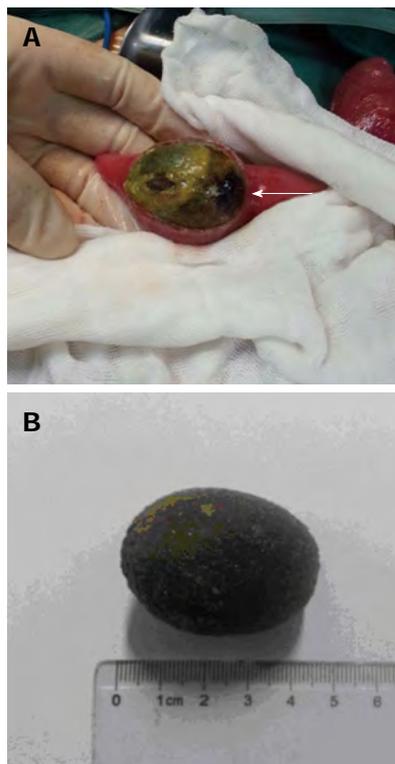
Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications<sup>[4]</sup>. Spontaneous closure of the fistulous tract is observed in  $> 50\%$  of cases<sup>[12]</sup>. Small-



**Figure 2 Severe air-fluid level.** A: Axial view of the upper abdomen demonstrates air in an intrahepatic bile duct (arrow); B: Axial view of the upper abdomen demonstrates an air in the gallbladder (arrow); C: Computed tomography demonstrating gallstones of approximately 5 cm in diameter (arrow) within the small bowel.

bowel obstruction requires enterolithotomy with a longitudinal incision placed on the anti-mesenteric border proximal to the site of impaction. Careful closure of the enterolithotomy is needed to avoid narrowing of the intestinal lumen, and we usually employ a transverse closure for this reason. The choice of surgical procedure is determined largely by clinical status. GI patients are usually elderly and have comorbidities so enterotomy with stone extraction alone appears to more suitable than more invasive techniques<sup>[4,16]</sup>.

However, 5% of patients who undergo enterolithotomy alone go on to develop biliary symptoms, and 10% require an unplanned reoperation. In the presence of residual stones, the estimated prevalence of recurrence ranged from 5% to 17%, and more than half of these recurrences occur within 6 mo of the index presentation.



**Figure 3** The gallstone was removed and the enterotomy repaired in two layers. A: Impacted stone (arrow) removed from the intestine; B: Gallstone measuring 3 cm × 4 cm × 5 cm.

Retrospective cohort and literature reviews of GI reveal a prevalence of biliary malignancy of 2%-6%<sup>[4,17]</sup>.

We noted that fistula closure, if conducted urgently or as an emergency during the initial procedure, was independently associated with a higher prevalence of mortality than enterotomy and stone extraction alone. The reason may be that elderly patients have multiple comorbidities and an edematous surrounding area. Bowel resection is sometimes necessary, particularly in the presence of a perforation.

Laparoscopy-assisted methods have been reported by Sarli *et al*<sup>[18]</sup>, who successfully treated three women with GI. Their patients made uneventful recoveries. However, laparoscopy is somewhat more challenging in cases of dilated and an edematous bowel<sup>[18,19]</sup>.

Some special types of GI, such as Bouveret's syndrome (stones impacting in the duodenum causing gastric outlet obstruction), and stones in the stomach or the colon are suitable for non-surgical therapeutic options in around 20% of the patients. For example laserlithotripsy in Bouveret's syndrome<sup>[20]</sup> or extracorporeal shock wave lithotripsy<sup>[21]</sup> or even only endoscopic extraction<sup>[22]</sup> may be a promising and fast therapeutic alternative.

Historically, wound infections and dehiscence have been cited as being the most common complications after surgery in 25% to 50% of GI cases. In contrast to what has been published so far, the most common post-operative complication is acute renal failure followed by urinary tract infection and wound infections. Gastrointes-

tinal complications related to anastomotic leaks and intra-abdominal abscesses are highest in patients undergoing enterotomy with fistula closure<sup>[3,4,12]</sup>.

If the gallbladder is preserved at the initial procedure, delayed cholecystectomy must be addressed. This is because 5% of patients who have undergone enterolithotomy alone go on to develop biliary symptoms, and the risk of patent fistula reflux and resulting biliary malignancy<sup>[3,4,12]</sup>. In conclusion, GI is a rare condition affecting mainly the older population with a female predominance. If GI occurs in elderly patients with comorbidities, the often vague, intermittent symptoms may delay the diagnosis by days. The advent of CT and MRI has made it easier to diagnose GI. Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications.

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## Photodynamic therapy for high-grade dysplasia of bile duct *via* a choledochoscope

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High-grade dysplasia; Choledochoscope

**Core tip:** Due to significant surgical trauma and a low risk of canceration, surgeons face a dilemma regarding the decision to perform pancreaticoduodenectomy for high-grade dysplasia of the distal bile duct. This report is the first to describe the successful treatment of high-grade dysplasia of the distal bile duct using photodynamic therapy *via* a choledochoscope. This clinical case demonstrated that photodynamic therapy *via* a trans-T-tube choledochoscope may be an effective and promising protocol for carcinoma *in situ* or high-grade dysplasia of the distal common bile duct.

Zhou JJ, Xiong L, Li QL, Gu Y, Wen Y, Deng XF, Miao XY. Photodynamic therapy for high-grade dysplasia of bile duct *via* a choledochoscope. *World J Gastroenterol* 2013; 19(33): 5590-5592 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5590.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5590>

### Abstract

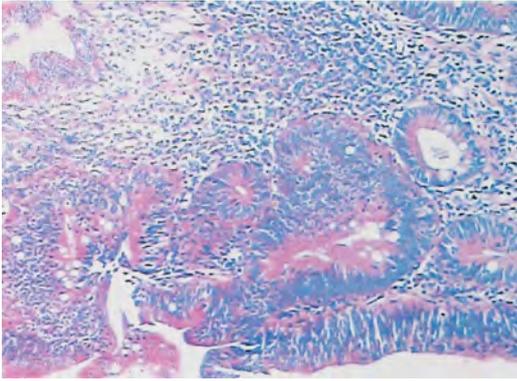
When a distal common bile duct neoplasm is at the stage of carcinoma *in situ* or high-grade dysplasia, it is difficult for the surgeon to decide whether to perform pancreaticoduodenectomy. Here we describe a patient with a progressive dysplastic lesion in the common bile duct, which developed from moderate-high to high-grade dysplasia in approximately 2 mo. The patient refused major surgery. Therefore, endoscopic-assisted photodynamic therapy was performed. The result at follow-up using a trans-T-tube choledochoscope showed that the lesion was completely necrotic. This report is the first to describe the successful treatment of high-grade dysplasia of the distal bile duct using photodynamic therapy *via* a choledochoscope.

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**Key words:** Photodynamic therapy; Common bile duct;

### INTRODUCTION

Endoscopic treatment is an alternative treatment option with low morbidity. Premalignant lesions such as high-grade dysplasia are being treated increasingly *via* endoscopy. Furthermore, as a promising clinical protocol, endoscopic-assisted photodynamic therapy may result in a more precise effect due to its selective damage to tumor cells. Saleem *et al*<sup>[1]</sup> reported successful photodynamic treatment (PDT) *via* endoscopic retrograde cholangiopancreatography (ERCP) for a villous adenoma with high-grade dysplasia. Here we report a unique case of progressive dysplasia in the distal common bile duct, which developed from moderate-high to high-grade dysplasia in approximately 2 mo, and was successfully treated by PDT *via* a choledochoscope.



**Figure 1** Pathological findings showed moderate-high grade dysplasia of the distal common bile duct lesion (hematoxylin/eosin staining,  $\times 100$ ).

## CASE REPORT

A 47-year-old man with previous common bile duct exploration and T-tube drainage for obstructive jaundice and poor general condition was admitted for follow-up examination. The patient had undergone surgery approximately 2 mo previously, which revealed an easily bleeding lesion of 3 cm in diameter occupying the distal common bile duct, and the pathological findings showed moderate-high grade dysplasia (Figure 1).

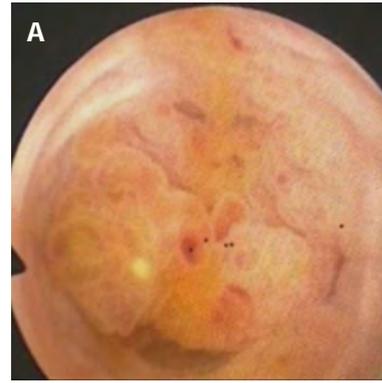
Choledochoscopy showed the same easily bleeding lesion as before (Figure 2A), and a second endoscopic biopsy revealed villous adenoma-like epithelia with moderate-high dysplasia and focal high-grade dysplasia. A trans-T-tube cholangiogram was performed, which demonstrated a dilated biliary tree with an irregular filling defect in the distal common bile duct (Figure 3).

The patient refused major surgery. Therefore, endoscopic-assisted PDT was performed. Informed consent was obtained from the patient prior to PDT. Hematoporphyrin (5 mg/kg) was administered intravenously 48 h before PDT. According to the surgical record and a previous computer tomography (CT) scan, we performed PDT through the T-tube sinus tract using a 4-cm long, cylindrical light diffuser at a power output of 250 mW/cm<sup>2</sup> in one application for a total dose of 70 J/cm<sup>2</sup>. This covered the whole distal common bile duct area. We noticed that the lesion became purple soon after light irradiation (Figure 2B). The patient felt well after the procedure and was discharged the next day.

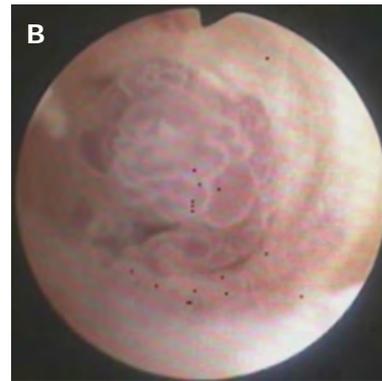
Two weeks later, trans-T-tube choledochoscopy showed that the lesion was completely necrotic (Figure 2C). The patient remained symptom-free, and at repeat choledochoscopy 3 mo later, no intraductal lesion was seen (Figure 2D). Furthermore, the video showed a functional duodenal papilla. The latest follow-up by choledochoscopy showed that the distal common bile duct was patent and the patient was asymptomatic and in good condition in March 2013.

## DISCUSSION

Tumors of the distal common bile duct present a serious



2012/8/1 Choledochoscopy finding: easily bleeding tumor in the lower extreme of common bile duct.



2012/8/13 Choledochoscopy view: the tumor 10 min after PDT.



2012/8/27 Two weeks after PDT: necrosis of the tumor.



A new bile duct with function observed 3 mo after PDT.

**Figure 2** Images taken *via* the trans-T-tube choledochoscope. A: Before photodynamic treatment (PDT); B: Ten minutes after PDT; C: Two weeks after PDT; D: Three months after PDT.

surgical problem because they necessitate a complicated and extensive resection. Pancreaticoduodenectomy (the Whipple procedure) is one of the most difficult procedures in general surgery, but should only be considered if the tumor is locally contained when identified. However, these small lesions tend to present late in the disease



**Figure 3 Trans-T-tube cholangiogram.** This image shows the dilated common bile duct and intrahepatic bile duct with an irregular filling defect in the distal common bile duct.

course, and many are unresectable<sup>[2]</sup>. Unfortunately, these lesions are not sensitive to chemotherapy or radiotherapy. In addition, studies have shown that intrahepatic bile duct hyperplasia is a potent precursor of cholangiocellular carcinoma. Livers with cholangiocellular carcinoma are closely associated with atypical hyperplasia and carcinoma *in situ*<sup>[3]</sup>. Therefore, when they are at the stage of carcinoma *in situ* or high-grade dysplasia, a new set of management dilemmas occur with regard to major surgery.

With more attention being paid to earlier detection and more careful pathological assessment, these previously overlooked histological features are attracting more interest not only in the diagnosis but also in the treatment.

Endoscopic surgery is an alternative treatment option with low morbidity in many cases<sup>[4]</sup>. Endoscopic-assisted photodynamic therapy is a promising method for lesions in the biliary tree due to the thickness of the bile duct wall being within the penetration depth of the light. PDT can be regarded as a standard palliative therapy for

unresectable cholangiocarcinoma as reported in previous studies<sup>[5]</sup>. In our case, it demonstrated high selectivity for the dysplastic lesion, and did not damage the duodenal papilla. This indicates the potential for endoscope-assisted PDT to provide precise ablation of intraductal lesions with progressive dysplasia. This protocol may serve as a standard modality for patients who are not candidates for surgery, and the T-tube sinus provides a good tract for both treatment and follow-up.

## ACKNOWLEDGMENTS

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## GENERAL INFORMATION

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The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

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*In press*

- Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- Geraud G**, Spierings EL, Keywood C. Tolerability and

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- Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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**Electronic journal (list all authors)**

- Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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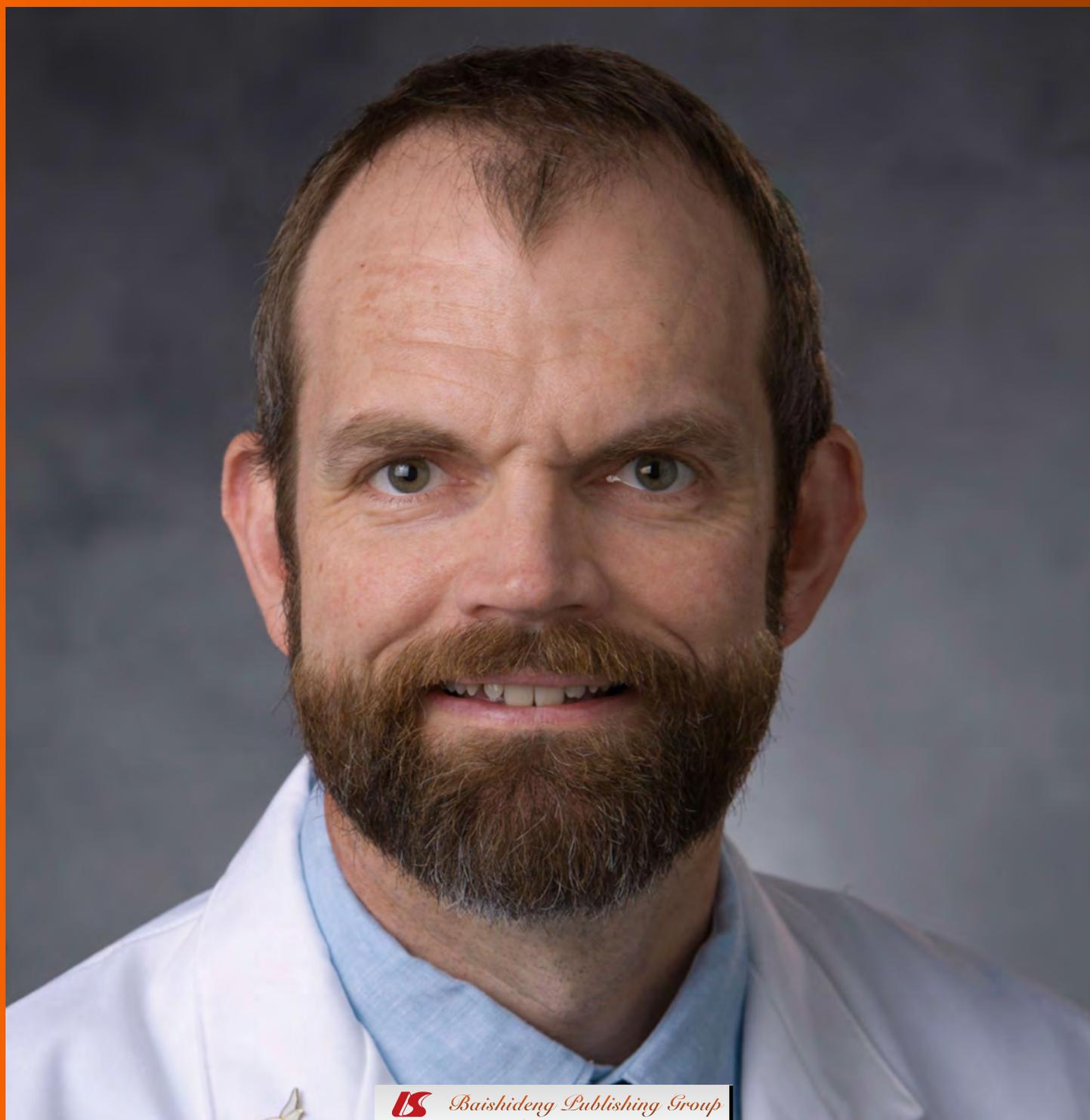
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## Confocal laser endomicroscopy in inflammatory bowel diseases: Dream or reality?

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### Dysplasia

**Core tip:** This paper reviews the current data on the clinical application of confocal laser endomicroscopy (CLE) in the study of colonic mucosa in patients with inflammatory bowel diseases (ulcerative colitis and Chron's disease). Moreover, the use of CLE has in diagnosing a biliary dysplasia/neoplasia in patients with primary sclerosing cholangitis, is evidenced.

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### Abstract

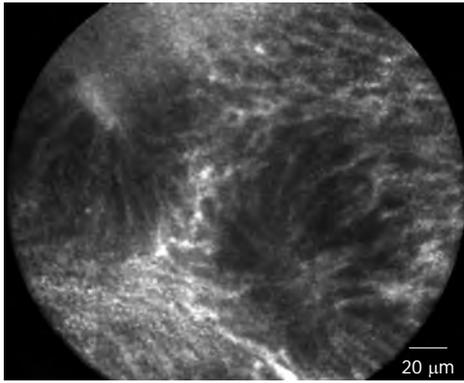
Confocal laser endomicroscopy (CLE) is a newly introduced procedure that provide real-time, high-resolution imaging of the gastrointestinal mucosa during endoscopy, allowing the visualization of the pathology of the mucosal epithelium with its cellular and subcellular structures. Recently, the use of CLE was reported in the study of colonic mucosa in patients with inflammatory bowel diseases and in particular in patients affected by ulcerative colitis. CLE has the potential to have an important role in management of inflammatory bowel diseases (IBD) patients as it can be used to assess the grading of colitis and in detection of microscopic colitis in endoscopically silent segments. Moreover, CLE can be used in surveillance programs especially in high-risk patients. Finally, CLE has been effectively used in diagnosing a biliary dysplasia/neoplasia in patients with primary sclerosing cholangitis, a pathological condition frequently associated with IBD, with a coexisting bile duct stricture.

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**Key words:** Inflammatory bowel diseases; Endoscopy; Confocal laser endomicroscopy; Colon cancer;

### CONFOCAL LASER ENDOMICROSCOPY

Confocal laser endomicroscopy (CLE) is a newly introduced procedure which allows to capture the images of "virtual histology" of the gastrointestinal mucosa during endoscopy<sup>[1,2]</sup>, so offering the opportunity to get the "real time" visualization of the pathology of the mucosal epithelium with its cellular and subcellular structures<sup>[3,4]</sup>. At present, CLE can be performed with 2 devices: one integrated into an endoscope (Pentax, Tokio, Japan, herein termed e-CLE) and one as a mini-probe through the scope (p-CLE; Cellvizio, Mauna Kea Technologies, Paris, France). Confocal microscopy consists of focusing a laser ray onto the mucosal surface and filtering the returned light by means of a small pinhole which rejects out-of-focus light. The illumination and detection systems are in the same focal plane and are termed "confocal". After passing the pinhole, the fluorescent light is detected by a photo-detection, transforming the light signal into an electrical one that is recorded by a computer. All detected signals from the illuminated spot are captured and measured. As the laser scans over the plane of interest, a whole



**Figure 1** Rectal mucosa of patient in remission from ulcerative colitis. Irregular alignment of crypt, crypt distortion and fusion with reduced amount of goblet cells.

image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light. The gray-scale image created is an optical section representing one focal plane within the examined specimen. Real-time confocal laser scanning microscopy-sequences (duration 1 min are recorded and stored digitally for later evaluation. CLE evaluation and its relative high-quality images have shown high agreement with the real histology of the tissue, so opening a wide spectrum of potential applications, all focused on the possibility of reducing and/or targeting the biopsies during endoscopy<sup>[5,6]</sup>. The current potential indications for CLE imaging are broad and include almost all the cases in which endoscopic biopsy is needed.

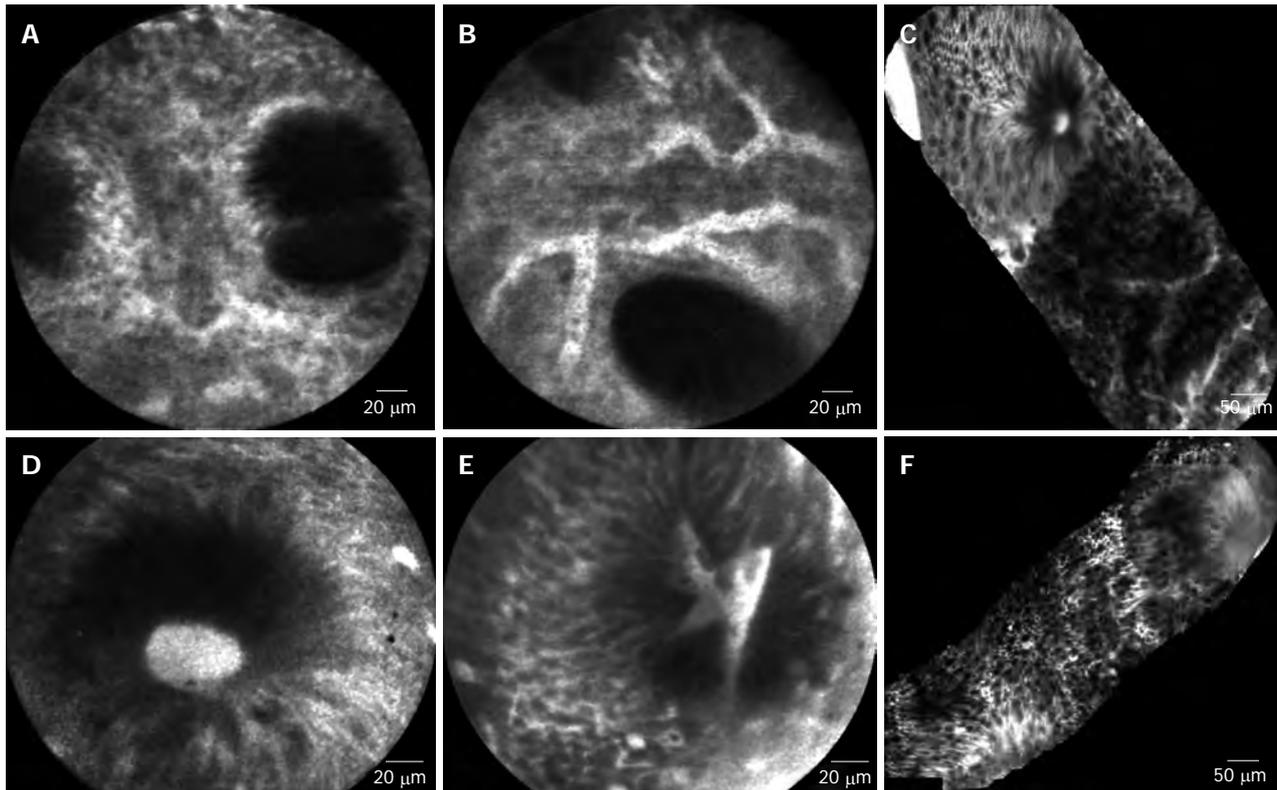
At now, various studies have addressed the potential usefulness of CLE in diagnostic work-up of inflammatory bowel disease (IBD), with particular interest to ulcerative colitis (UC)<sup>[7-9]</sup>. In effect, all the studies concerning the application of CLE in the UC context have shown that this technique can have a potential role in assessing the extension and the activity of disease and in targeting biopsies, reducing the number of useless biopsies and improving the early detection of dysplasia<sup>[10-13]</sup>. In the field of UC, the most frequent alterations in crypt architecture are represented by dilation of crypt openings, more irregular arrangement of crypts, enlarged spaces between crypt, crypt destruction and/or crypt fusion, and crypt abscess with fluorescein leaks into the crypt lumen (therefore making the lumen brighter than the surrounding epithelium). Microvascular alterations are mainly represented by dilated, prominent branching vessels (Figures 1 and 2). The study by Watanabe *et al*<sup>[14]</sup>, including 17 patients with active UC compared with 14 controls, showed that CLE images provided equivalent information to histopathology with respect to definition of the main histological outcomes (crypt architecture, capillaries and inflammatory cells). On these bases, a new classification of inflammatory activity in UC using CLE has been proposed<sup>[15]</sup>, which comprises the assessment of crypt architecture, microvascular alterations and fluores-

cein leakage.

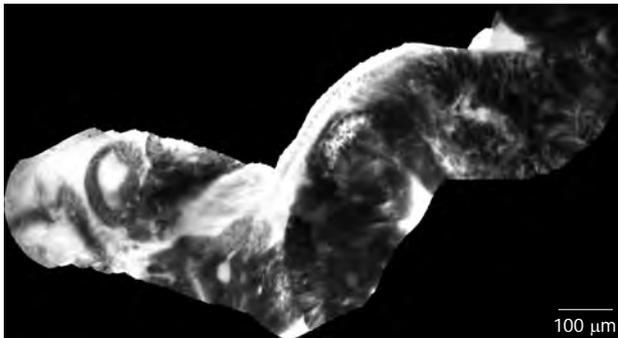
One of the most important diagnostic goals in the management of patients with UC, especially of those who present risk factors for cancer development, should be the “real-time” endoscopic identification and diagnosis of dysplasia/neoplasia, as this would reduce the number of unnecessary biopsies with their associated time and costs<sup>[16,17]</sup>. Starting from these assumptions, Kiesslich *et al*<sup>[18]</sup> have shown for the first time that the diagnosis of dysplasia/neoplasia in UC could be maximized by using both pan-chromoendoscopy (CE) and targeted CLE, with high values of diagnostic accuracy (sensitivity 94%, specificity 98%). This result has been recently confirmed, although with less remarkable diagnostic values, by van den Broek *et al*<sup>[19]</sup>, who reported a diagnostic accuracy of 81% when comparing CLE with narrow-band imaging plus high-definition endoscopy (diagnostic accuracy 92%). In accordance with these reports, a recent paper produced by our group, exploring the efficacy of the combined application of CE and targeted p-CLE in diagnosing dysplasia in longstanding UC in the “real-life”, has underlined the high diagnostic accuracy of such a procedure compared to standard histology (sensitivity 100%, specificity 90%, positive predictive value 83% and negative predictive value 100%)<sup>[20]</sup> (Figure 3). Giving value to all the above-mentioned contributions, the combination of CE and CLE corresponds to a diagnostic gain of 3- to 5-fold for detecting dysplasia/neoplasia than conventional colonoscopy<sup>[21]</sup>. The diagnostic gain is mainly due to CE application which could dramatically decreased (about of 10 times) the number of biopsies when just circumscribed suspicious lesions on CE would have been targeted; if only CLE-suspected neoplastic lesions had undergone biopsy after CE, the mean number of biopsies would be further reduced.

More recently, Neumann *et al*<sup>[22]</sup> have explored the clinical utility of CLE also in 76 patients affected by Crohn’s disease (CD), particularly determining whether the disease activity can be graded by using CLE. In effect, a relevant percentage of patients with active CD presented an increased colonic crypt tortuosity, enlarged crypt lumen, microerosions, augmented vascularization and increased cellular infiltrates. Starting from these considerations, these authors proposed a CLE score for assessing CD activity *in vitro*, with such a score having of potential utility for predicting the course of CD and the response to medical therapy.

CLE application in IBD has been evaluated even under a prognostic view. A nice work by Kiesslich *et al*<sup>[23]</sup> have shown that “cell shedding” and “barrier loss” detected by CLE are able to predict relapse of IBD and have potential role as diagnostic tool for the management of the disease. In this paper, the sensitivity, specificity and accuracy for the “CLE grading system” to predict a flare were 62.5%, 91.2% and 79%, respectively. Interestingly, a recent paper by Turcotte *et al*<sup>[24]</sup> confirmed the high prognostic power of CLE in predicting the course for other relevant clinical end-points for patients affected by IBD.



**Figure 2** Colonic mucosa. A: Patient in remission from ulcerative colitis. Crypt distortion and fusion and many capillaries visible in the lamina propria; B: Patient in remission from ulcerative colitis. Enlarged spaces between crypts and dilated prominent branching vessels; C: In patient with active ulcerative colitis (distal colitis). Image of colonic mucosa showing the switch from normal mucosa (top of the figure) to inflamed mucosa. Inflamed mucosa showing irregular arrangement of crypts, crypt fusion and capillaries alterations; D: In patient with active ulcerative colitis. Dilated and bright crypt lumen (fluorescein leakage) with intact epithelium; E: In patient with active ulcerative colitis. Dilated, irregular and bright crypt lumen (fluorescein leakage) with partially intact epithelium; F: In patient with highly active ulcerative colitis (Mayo CU3). Crypts distortion and destruction, crypt abscess and crypts replacement by diffuse necrosis.



**Figure 3** Dysplasia-associated lesional mass in long-standing ulcerative colitis. Image of colonic mucosa evidencing the switch from the inflamed mucosa, to the neoplastic mucosa. Inflamed mucosa is characterized by crypts fusion and distortion, dilation of crypt openings, enlarged spaces between crypts, and microvascular alterations with fluorescein leaks into the crypt lumen. Dysplastic mucosa (right corner) is characterized by "dark" cells, irregular architectural patterns with villiform structures and a "dark" epithelial border.

In particular, increased epithelial gaps in the small intestine as determined by CLE were a predictive factor for future hospitalization or surgery in IBD patients.

Going to another potential indication of this procedure in diagnostic work-up of IBD patients, CLE has been effectively used in diagnosing a biliary dysplasia/neoplasia in patients with primary sclerosing cholangitis

(PSC), a pathological condition frequently associated with IBD, with a coexisting bile duct stricture<sup>[25]</sup>. In effect, Heif *et al*<sup>[25]</sup> showed a high diagnostic accuracy in detecting the presence of a bile duct neoplasia in 15 PSC patients with 21 dominant stenoses (sensitivity 100%; specificity 61%; positive predictive value 22%; negative predictive value 100%). This paper has opened the doors to a further potential application of CLE in IBD.

Unfortunately, some relevant limitations reduced the current application of CLE in general practice: the need for a learning curve, the cost of the equipment, the need for an extra-time (about 30 min) to enhanced colonoscopy and, not less important, a number of medical-legal issues. Furthermore, the promising results in the literature derived from a little number of trials and still from a few experienced centers and, as a consequence, these cannot be generalized easily.

In our mind, among these limitations, the need for an adequate learning curve represents the less relevant topic. In effect, as shown in previous papers, the operator's endoscopic expertise and learning curve represent the crucial issues and main limitation for the routine application of this endoscopic technique. However, a recent report has highlighted that the ability to accurately interpret CLE images for predicting neoplastic lesions can be learned rapidly by a range of GI specialists<sup>[26]</sup>; similarly,

the ability to acquire high-quality CLE images can also be learned quickly.

About medical-legal issues, mainly regarding the application of CLE for surveillance endoscopy in UC, the principal matter is represented by the fact that endoscopists would make a histological diagnosis without the confirmation by a pathologist and would decide during the endoscopy if performing or not multiple biopsies. At now, this kind of diagnostic approach is not reported by the current guidelines<sup>[27]</sup> and should be applied only within a controlled trial formally approved by an ethical committee.

Concluding, new multicenter studies are needed to assess the real cost-effectiveness of CLE technique for IBD. In our opinion, when balancing the interesting diagnostic advantage of CLE in clinical practice with its important realistic limitation, the wide application of this procedure in the current endoscopic practice still appears to be more a dream than a reality.

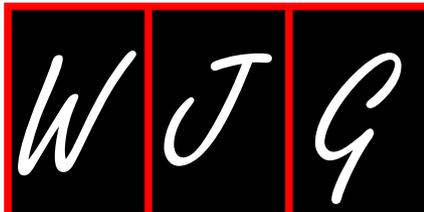
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## Epidemiology of esophageal cancer

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### Abstract

Esophageal cancer (EsC) is one of the least studied and deadliest cancers worldwide because of its extremely aggressive nature and poor survival rate. It ranks sixth among all cancers in mortality. In retrospective studies of EsC, smoking, hot tea drinking, red meat consumption, poor oral health, low intake of fresh fruit and vegetables, and low socioeconomic status have been associated with a higher risk of esophageal squamous cell carcinoma. Barrett's esophagus is clearly recognized as a risk factor for EsC, and dysplasia remains the only factor useful for identifying patients at increased risk, for the development of esophageal adenocarcinoma in clinical practice. Here, we investigated the epidemiologic patterns and causes of EsC. Using population based cancer data from the Surveillance, Epidemiology and End Results Program of the United States; we generated the most up-to-date stage distribution and 5-year relative survival by stage at diagnosis for 1998-2009. Special note should be given to the fact that esophageal cancer, mainly adenocarcinoma, is one of the very few cancers that is contributing to increasing death rates (20%) among males in the United States. To further explore the mechanism of development of EsC will hopefully decrease the incidence of EsC and improve outcomes.

**Key words:** Risk factor; Barrett's esophagus; Cyclin D1 G870A; Esophageal neoplasm; Susceptibility; Polymorphism

**Core tip:** Here, we investigated the epidemiologic patterns and causes of esophageal cancer. Using population based cancer data from the Surveillance, Epidemiology and End Results Program of the United States; we generated the most up-to-date stage distribution and 5-year relative survival by stage at diagnosis for 1998-2009.

Zhang Y. Epidemiology of esophageal cancer. *World J Gastroenterol* 2013; 19(34): 5598-5606 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5598.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5598>

### INTRODUCTION

Esophageal cancer (EsC) including squamous cell carcinoma (SCC) and adenocarcinoma is considered as a serious malignancy with respect to prognosis and a fatal outcome in the great majority of cases<sup>[1,2]</sup>. Esophageal carcinoma affects more than 450000 people worldwide and the incidence is rapidly increasing<sup>[3]</sup>. Currently, EsC is the eighth most common incident cancer in the world because of its extremely aggressive nature and poor survival rate<sup>[4,5]</sup>.

EsC exhibits an epidemiologic pattern distinct from all other cancers<sup>[6,7]</sup>. The incidence of esophageal adenocarcinoma has increased sharply over the past few decades, both by period and birth cohort. Etiological studies are required to explain the rapid increase of this lethal cancer<sup>[8]</sup>. Understanding the epidemiology of EsC will be the key to elucidating the causes and risk factors for esophageal cancer and thus the cornerstone of developing any prevention strategies.

### PATHOLOGY AND ANATOMY

Cancer of the esophagus typically occurs in one of two

forms, SCCs arising from the stratified squamous epithelial lining of the organ, and adenocarcinomas affecting columnar glandular cells that replace the squamous epithelium<sup>[9]</sup>. Sarcomas and small cell carcinomas generally represent less than 1%-2% of all esophageal cancers<sup>[10,11]</sup>. On rare occasions, other carcinomas, melanomas, leiomyosarcomas, carcinoids, and lymphomas may develop in the esophagus as well<sup>[5]</sup>.

SCC is the predominant histologic type of esophageal cancer worldwide<sup>[12]</sup>. The incidence of squamous cell cancer of the esophagus increases with age as well and peaks in the seventh decade of life. The incidence of squamous cell esophageal cancer is three times higher in blacks than in whites, whereas adenocarcinomas are more common in white men.

The natural histories of SCCs and adenocarcinomas of esophagus appear to differ substantially. For squamous cell cancers, transition models have described squamous epithelium undergoing inflammatory changes that progress to dysplasia and in situ malignant change<sup>[13,14]</sup>.

Most adenocarcinomas, however, tend to arise in the distal esophagus from columnar-lined metaplastic epithelium, commonly known as Barrett's esophagus<sup>[15,16]</sup>, which replaces the squamous epithelium during the healing reflux esophagitis and may progress to dysplasia. Gastroesophageal reflux disease (GERD), or just reflux<sup>[17-19]</sup> can damage the lining of esophagus which causes Barrett's esophagus<sup>[17]</sup>, characterized by abnormal "tongues" of salmon-colored mucosa extending proximally from the gastroesophageal junction into the normal pale esophageal mucosa, develops in approximately 5 to 8 percent of patients with gastroesophageal reflux disease.

Cancers that start at the area where the esophagus joins the stomach (the GE junction), which includes about the first 2 inches of the stomach (called the cardia), tend to behave like esophagus cancers (and are treated like them, as well), so they are grouped with esophagus cancers. Approximately three quarters of all adenocarcinomas are found in the distal esophagus, whereas SCCs are more evenly distributed between the middle and lower third. The cervical esophagus is an uncommon site of disease. Nowadays the terminology used for the definition of adenocarcinomas at the GE junction is "cardiac carcinoma", which can be easily misunderstood. This definition of adenocarcinomas of the GE junction does not allow correct comparison of diagnosis (endoscopic, radiological and pathologic), epidemiology and surgical therapy in national and international aspects, because different tumor can develop in the same area, and all called cardia tumors<sup>[20]</sup>. Siewert and Stein recommended a classification to solve this problem<sup>[21]</sup>. The classification of the tumors is morphological/topographical<sup>[21,22]</sup>. Type I is adenocarcinoma of the distal part of the esophagus. Type II is adenocarcinoma of the real cardia and type III is subcardial gastric adenocarcinoma. The importance of this classification is it enables unified pre-operative assessment and it can also help to decide the type of the surgical intervention<sup>[20,23-27]</sup>.

## INCIDENCE

Cancers arising from the esophagus, including the GE junction, are relatively uncommon in the United States<sup>[28,29]</sup>. The rate of cancer of the distal esophagus is about equal to that of the more proximal two-thirds<sup>[30]</sup>. SCC is the predominant histologic type of esophageal cancer worldwide. The incidence of SCC increases with age as well and peaks in the seventh decade of life, which is three times higher in blacks than in whites, whereas adenocarcinomas are more common in white men.

The most important precancerous disease is Barrett's esophagus<sup>[31-34]</sup>. Patients with Barrett's esophagus have a 50 to 100 times increase in their risk of developing cancer compared to the general population. People with Barrett's esophagus are much more likely to develop cancer of the esophagus. These people require close medical follow-up in order to find cancer early. Still, although they have a higher risk, most people with Barrett's esophagus do not go on to develop cancer of the esophagus. In their population-based cohort study, Hvid-Jensen *et al*<sup>[35]</sup> reported an annual risk of esophageal adenocarcinoma of 0.12% among patients with Barrett's esophagus.

For different types of esophageal cancer, the risk increases with age, with a mean age at diagnosis of 67 years. Esophageal cancer age-adjusted incidence of blacks was about twice that of whites (8.63/100000 *vs* 4.39/100000,  $P < 0.05$ )<sup>[36]</sup>. Squamous cell carcinoma was more commonly diagnosed in blacks and white females, whereas adenocarcinoma was more common among white males.

Although the disease is relatively uncommon in the United States, it is a major global health threat<sup>[37]</sup>. Esophageal cancer is four times more common and slightly more lethal in men than in women. According to the National Cancer Institute (Cancer.gov) in 2012, it is estimated that 17460 persons (13950 men and 3510 women) will be diagnosed with and 15070 persons will die of cancer of the esophagus in 2012.

Esophageal cancer occurs at a rate 20 to 30 times higher in China than in the United States. An esophageal "cancer belt," primarily squamous cell cancers, extends from northeast China to the Middle East<sup>[38-40]</sup>. Evidence of an association between environment and diet and esophageal cancer comes from the profound differences in incidence observed in various parts of the world. The majority of the factors so far implicated in cancer of the esophagus appear to act directly on the esophagus rather than systemically. Nutritional deficiencies can develop by chronic alcohol use as well as by poverty and lack of an adequate food supply, but diet does not explain the whole picture. External carcinogens are necessary to affect the end result. The association between nutrition and esophagitis may suggest methods of primary prevention of esophageal cancer and provide a chance of lowering the incidence of this deadly disease<sup>[31]</sup>.

From 1996-2009, the annual percentage change was increased by 0.5% in all races and 0.4% in white. How-

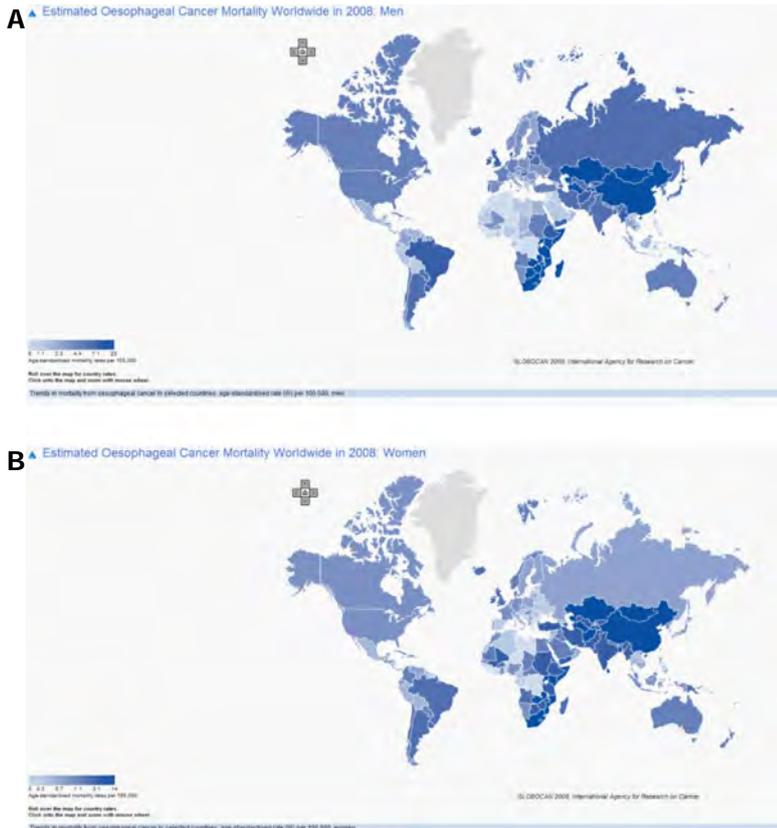


Figure 1 Estimated esophageal cancer mortality worldwide in 2008 (GLOBOCAN 2008). A: Men; B: Women.

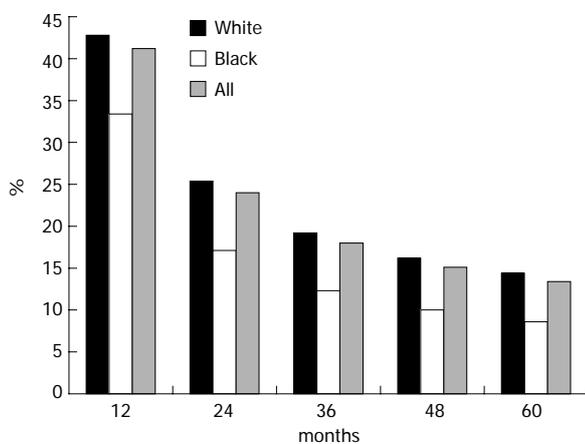


Figure 2 Relative survivals for esophageal cancer for all races.

ever, the increase of incidence is because of the increase incidence in men. Actually, the incidence in woman dropped by 0.4% (Surveillance, Epidemiology and End Results, SEER).

## MORTALITY

Figure 1 shows the age-adjusted esophageal cancer mortality. It is in line with the incidence rate in the world but there is no difference between men and women. Age-adjusted mortality for blacks, although showing a declining trend, was nearly twice that of whites (7.79 vs 3.96,  $P < 0.05$ ). Squamous cell carcinoma was more commonly diagnosed in blacks and white females, whereas adeno-

carcinoma was more common among white males ( $P < 0.001$ )<sup>[41]</sup>. The reasons are economic status, diet, and poor eating habits, *etc.*

## SURVIVAL

Survival varied widely according to cancer site. The differences in survival related to histology were also expected<sup>[42]</sup>. Although survival was poor for all groups, it was significantly poorer in blacks than in whites (Figure 2). The overall 5-year relative survival for 2002-2008 from 18 SEER geographic areas was 16.9%. Five-year relative survival by race and sex was: 18.1% for white men; 17.0% for white women; 10.4% for black men; 12.6% for black women.

The overall relative 5-year survival rates over time increase gradually in white and black, man and women. For example, the rate was below 2% in 1995 to over 10% in 2008 in black men (SEER).

Although the overall outlook for patients diagnosed with esophageal cancer has improved in the past 30 years, most patients still present with advanced disease, and their survival remains poor<sup>[43]</sup>. One-third to one-half of patients treated with either chemoradiation therapy or chemoradiation therapy plus surgery are alive at 2 years, without recurrence of esophageal cancer.

The reason is because esophageal cancer is diagnosed at rather late stage. Overall, more than 30 percent of patients have metastatic disease at the time of presentation (32.15% in white and 31.83% in black). None was found that has in situ cancer, due to the fact that it can be diffi-

**Table 1** Stage distribution and 5-year relative survival by stage at diagnosis for 1998-2009, all races, both sexes

Stage at diagnosis	Stage distribution	5-year relative survival
Localized (confined to primary site)	22%	37.80%
Regional (spread to regional lymphnodes)	30%	19.80%
Distant (cancer has metastasized)	35%	3.40%
Unknown (unstaged)	13%	10.50%

cult to diagnose esophageal cancer early. Among patients who are undergoing primary surgery, 22 percent have localized disease, 30 percent have regional cancer (Table 1).

## RISK FACTORS

The patterns of esophageal cancer are dramatically changing in the United States. However, the mechanisms of esophageal tumorigenesis are not fully understood<sup>[5]</sup>. Three decades ago the large majority of these cancers were SCCs, but the incidence of esophageal adenocarcinoma has been steadily increasing<sup>[44]</sup>. Tobacco and alcohol consumption are the primary causes of SCCs of the esophagus<sup>[45]</sup>. One of the strongest emerging risk factors, however, is obesity. Increases in the prevalence of obesity and the incidence of esophageal adenocarcinoma are parallel, and several epidemiologic studies have shown upwards of threefold excess risks among overweight individuals. Further research into the causes of these usually fatal cancers may help identify other potential determinants and provide needed information to help stem their increase.

Cigarettes, red meat, alcohol and hookah smoking<sup>[4]</sup>, nass use (a chewing tobacco product), opium consumption, hot tea drinking, poor oral health, low intake of fresh fruit and vegetables, and low socioeconomic status have been associated with a higher risk of esophageal SCC (Table 2). Barrett's esophagus is clearly recognized as a risk factor for EsC, and dysplasia remains the only factor useful for identifying patients at increased risk, for the development of esophageal adenocarcinoma in clinical practice.

### Smoking increases risk of SCC and adenocarcinoma of the esophagus

Moderate to heavy smokers face an increased risk of both SCC and adenocarcinoma of the esophagus. Research suggests that when a smoker ingests tobacco condensates, it causes tobacco carcinogens, particularly nitrosamines, to come in contact with the esophageal mucosa. There is a direct correlation between the number of cigarettes a smoker smokes per day; the length of time the smoker spends smoking, and the risk of esophageal cancer<sup>[2]</sup>.

### The effects of chronic irritation and inflammation on SCC

The incidence of SCC of the esophagus has been found to dramatically increase in the presence of any factor that causes chronic irritation and inflammation, such as exces-

**Table 2** Esophageal cancer risk factors<sup>[5,80-87]</sup>

Risk factor	Squamous-cell carcinoma	Adenocarcinoma
First or second hand smoke	+++	++
Alcohol consumption	+++	-
Consumption of red meat	+	+
Barrett's esophagus	-	++++
Reflux symptoms	-	+++
Being overweight	-	++
Poverty	++	-
Caustic injury to the esophagus	++++	-
History of head and neck cancer	++++	-
History with radiotherapy	+++	+++
Frequent consumption of extremely hot drinks	+	-
Polymorphism Cyclin D1 (CCND1)	-	+
G870A polymorphism		
p53 polymorphism	+	-
TERT A279T polymorphism	+	+

--: No effect; +: Suspicious effect; ++: Positive effect; +++, ++++: Strong positive effect.

sive alcohol intake, especially in combination with smoking<sup>[46,47]</sup>. This does not hold true for adenocarcinoma. This may account for more than 90 percent of all cases of SCC of the esophagus in developed countries<sup>[48]</sup>.

Chronic esophageal irritation also occurs when food is retained and decomposed by bacteria, releasing various chemical irritants. Frequent consumption of hot beverages also appears to increase the incidence of SCC<sup>[49]</sup>.

### Obesity

Esophageal squamous cell carcinoma (ESCC) is clearly linked to a low socioeconomic status. The increasing prevalence of obesity in the Western world is thought to add to the rising incidence of esophageal adenocarcinoma. More specifically, it has been postulated that obesity increases intraabdominal pressure and gastroesophageal reflux by a specific mechanism, although some studies provided contradictory results. On the other hand, adipose tissue itself influences tumor development<sup>[50-54]</sup>. Adipocytes and inflammatory cells secrete adipokines and cytokines which are known to promote tumor development. The abundant availability of lipids from adipocytes in the tumor microenvironment, supports tumor progression and uncontrolled growth. Given that adipocytes are a major source of adipokines and energy for the cancer cell, understanding the mechanisms of metabolic symbiosis between cancer cells and adipocytes, should reveal new therapeutic possibilities.

### Genetic changes

The genetic and molecular changes underlying the development of EsC remain poorly understood. Genetic analysis of these cancers reveals frequent chromosomal losses (4q, 5q, 9p, and 18q), chromosomal gains (8q, 17q, and 20q), and occasional gene amplifications (7, 8, and 17q)<sup>[5]</sup>.

In the past decade, efforts have been made to use can-

didate gene approaches to identify genetic susceptibility factors for ESCC. The genome-wide association studies (GWAS) has emerged as a powerful and successful tool to identify common disease alleles by using high-throughput genotyping technology to interrogate a large number of tagging single nucleotide polymorphisms (SNPs) that serve as surrogates for untested common SNPs across the genome. So far, GWAS of esophageal cancers including ESCC in individuals of European and Japanese ancestry, have shown that variants in *ADH* genes and/or *ALDH2* are associated with risk of ESCC<sup>[55-58]</sup>. More recently, Wu *et al* further reported that nine new ESCC susceptibility loci, of which seven, at chromosomes 4q23, 16q12.1, 17q21, 22q12, 3q27, 17p13 and 18p11, had a significant marginal effect ( $P = 1.78 \times 10^{-39}$  to  $P = 2.49 \times 10^{-11}$ ) and two of which, at 2q22 and 13q33, had a significant association only in the gene-alcohol drinking interaction [gene-environment interaction  $P (P_{G \times E}) = 4.39 \times 10^{-11}$  and  $P_{G \times E} = 4.80 \times 10^{-8}$ , respectively]. Variants at the 4q23 locus, which includes the *ADH* cluster, each had a significant interaction with alcohol drinking in their association with ESCC risk ( $P_{G \times E} = 2.54 \times 10^{-7}$  to  $3.23 \times 10^{-3}$ ). They confirmed the known association of the *ALDH2* locus on 12q24 to ESCC, and a joint analysis showed that drinkers with both of the *ADH1B* and *ALDH2* risk alleles had a fourfold increased risk for ESCC compared to drinkers without these risk alleles. Their results underscore the direct genetic contribution to ESCC risk, as well as the genetic contribution to ESCC through interaction with alcohol consumption<sup>[55]</sup>.

There are also some studies on polymorphism on other locations for esophageal adenocarcinoma with smaller samples. Cyclin D1 (*CCND1*) *G870A* polymorphism has been known to be a risk factor in multiple cancers<sup>[59-63]</sup>. However, investigations concerning the association of *CCND1 G870A* polymorphism with esophageal cancer risk have generated conflicting results<sup>[64-69]</sup>. The overall data suggest that *CCND1 G870A* variations might have an association with increased esophageal cancer susceptibility. The earliest findings, published in 2005, reported that *CCND1 G870A* was a risk factor for esophageal adenocarcinoma<sup>[67]</sup>. A study conducted by Liu's group, drew the exact opposite conclusion: *CCND1 G870A* was not associated with susceptibility to esophageal adenocarcinoma<sup>[70]</sup>. Liu's group explained the discrepancy by noting that all previous studies were based on small samplings.

Since the definition of *G870A* is the same for both groups, the significant difference lies in the methods that they used. Casson's group did polymerase chain reaction (PCR) followed by enzyme digestion, and visualized the result by running the products in a 15% acrylamide gel, and is referred to as "PCR-restriction fragment length polymorphism (RFLP)," which was widely used ten years ago. Liu's group genotyped by the 5'-nuclease assay (Taq-Man), using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, United States). This is currently considered the gold standard in genotyping. Casson's group included patients with

GERD. Since GERD is rather common in the general population, they selected strictly asymptomatic individuals for their control groups. Liu's group chose instead to use healthy visitors as their control group. And those healthy controls might have had some undiagnosed diseases related to GERD, such as Barrett's esophagus.

One source of bias between the two groups may lie in the different controls that were used. This may explain why the rate of G/G is different between the two groups. The second reason may be due to the detection method used. Usually, sequencing is viewed as the gold standard, but it is not always correct<sup>[71]</sup>. To detect polymorphism, the PCR-RFLP that Casson's group used, might have been a better choice because PCR-RFLP tests detect the correct genotype. Direct sequencing of the PCR products, obtained with one of the primers located adjacent to a mutated nucleotide, may cause unequal amplification of alleles in heterozygous samples. This effect is even stronger when mismatched primers are used. Therefore, there is a potential pitfall in DNA sequencing, indicating that sequencing may not always be the gold standard. The third reason may be due to the inherent differences between the two groups. As we know, the minor allele frequency (maf) of a SNP is different among different populations. Since it is ethnicity related, more information is needed to know the demographic information of the patient and the control group.

Although Li argues that others may be drawing different conclusions than his group, due to smaller samplings, it cannot be ruled out that other factors are involved, such as how the different control groups were recruited. Zhuo *et al*<sup>[64]</sup> reported that homozygous AA alleles might elevate esophageal cancer risk among Asians, but not Caucasians. This might partially explain why the two groups drew different conclusions.

*CCND1 G870A* polymorphism might be a low-penetrant risk factor for esophageal carcinoma, particularly among Asians. More information is needed to study large samples in relationship to pertinent demographic data.

## PREVENTIVE FACTORS

The keys to prevention of esophageal cancer vary by cell type. For SCC, reduction or elimination of tobacco and alcohol consumption provide the best means to reduce the incidence of this cancer. However, no one particular risk factor is responsible for the rising incidence of esophageal adenocarcinoma. Several preventive strategies are under investigation using such agents as nonsteroidal anti-inflammatory drugs, selenium, alpha-difluoromethylornithine, and retinoids<sup>[72]</sup>. Vegetable intake, and fruit intake is considered to be a preventive role. Carotene, vitamin C, and vitamin E are protective, most likely in combination with each other and other micronutrients. The role of vitamin A is not clear because of conflicting findings in the studies reviewed<sup>[73]</sup>. When intake of raw vegetables and cooked vegetables was analyzed separately, raw vegetables were found to be more protective.

Because fruits are relatively expensive in most places, increased consumption may reflect higher socioeconomic status.

Since obesity is closely related to the incidence of the esophageal cancer, it would be interesting to follow up those patients with precancerous lesion to monitor their weight.

In patients with high-grade dysplasia, the options for preventive approaches include surveillance, endoscopic therapies, and surgical resection, but the optimum approach is debated<sup>[5]</sup>. In an analysis of more than 15 studies, the mean incidence of occult adenocarcinoma in patients with a preoperative diagnosis of high-grade dysplasia treated with esophagectomy was 41%. This high incidence provides a rationale for use of esophagectomy, but there is concern about the risk of morbidity. Use of endoscopic treatments for high-grade dysplasia has been supported in two randomised trials. In one trial of photodynamic therapy plus proton-pump inhibitors compared with proton-pump inhibitors alone, progression to cancer was significantly decreased in the photodynamic-therapy group (13% *vs* 28%). In the other, which assessed endoscopic radiofrequency ablation in patients with Barrett's esophagus and high-grade dysplasia, radio frequency ablation was more effective in eradication of high-grade dysplasia than a proton-pump inhibitor alone, and the progression to cancer was lower (4% *vs* 22%) during short-term follow-up<sup>[74-77]</sup>.

## SCREENING AND EARLY DETECTION

Although several potential preventive measures exist, none has been proven to decrease the risk of esophageal carcinoma in prospective well-designed trials<sup>[5]</sup>. The relatively low incidence of esophageal cancer, the absence of early symptoms, and the rarity of a hereditary form of the disease make population-based screening untenable except in certain high-risk areas of the world<sup>[5]</sup>.

Patients who are found to have Barrett's esophagus, however, may be candidates for regular endoscopic surveillance, since the incidence of low-grade dysplasia, high-grade dysplasia, and cancer is approximately 4 percent, 1 percent, and 0.5 percent per year, respectively, among such patients<sup>[5]</sup>. Whether endoscopic screening programs to detect Barrett's esophagus in patients with chronic reflux disease symptoms are useful has been debated. Critics point out the high number of people in the general population who have reflux symptoms and the fact that at least 40% of patients with Barrett's esophagus do not have reflux symptoms, and question the cost-effectiveness of screening. Proponents of screening for Barrett's esophagus point to the clear associations between reflux, Barrett's esophagus, and esophageal adenocarcinoma, and suggest that the rising incidence of esophageal adenocarcinoma justifies screening. No definitive data are available on whether endoscopic screening for Barrett's esophagus is associated with a reduction in cancer-related mortality and, therefore, screening is

not routinely recommended.

However, some experts have recommended that endoscopy be performed every three to five years in patients who have Barrett's esophagus in the absence of epithelial dysplasia and more frequently if they are found to have low-grade dysplasia. Diagnostic endoscopy for early detection can be conducted in 2 steps: at first detection of an abnormal area through changes in relief, in color or in the course of superficial capillaries; then characterization of the morphology of the lesion. Then treatment decision offers 3 options according to histologic prediction: abstention, endoscopic resection, surgery. The rigorous quality control of endoscopy will reduce the miss rate of lesions and the occurrence of interval cancer<sup>[78]</sup>.

## CONCLUSION

The precise causes of EsC have not been identified. Despite uncertainties in our understanding of the causes of mechanistic pathways of esophageal cancer, there is sufficient evidence to take effective steps to prevent the majority of SCC in western countries, while more information is needed to curb the epidemic increase in adenocarcinoma<sup>[7,79]</sup>.

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## Appendectomy and *Clostridium difficile* colitis: Relationships revealed by clinical observations and immunology

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### Abstract

Advances in understanding the interaction between the human immune system and the microbiome have led to an improved understanding of the function of the vermiform appendix as a safe-house for beneficial bacteria in the colon. These advances have been made despite long standing clinical observations that the appendectomy is a safe and effective procedure. However, more recent clinical data show that an appendectomy puts patients at increased risk for recurrent *Clostridium difficile* (*C. difficile*)-associated colitis, and probably other diseases associated with an altered microbiome. At the same time, appendectomy does not apparently put patients at risk for an initial onset of *C. difficile*-associated colitis. These clinical observations point toward the idea that the vermiform appendix might not effectively protect the microbiome in the face of broad spectrum antibiotics, the use of which precedes the initial onset of *C. difficile*-associated colitis. Further, these observations point to the idea that historically important threats to the microbiome such as infectious gastrointestinal pathogens have been supplanted by other threats, particularly the use of broad spectrum antibiotics.

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**Key words:** Appendectomy; *Clostridium difficile*; Colitis; Diarrheal illness; Vermiform appendix

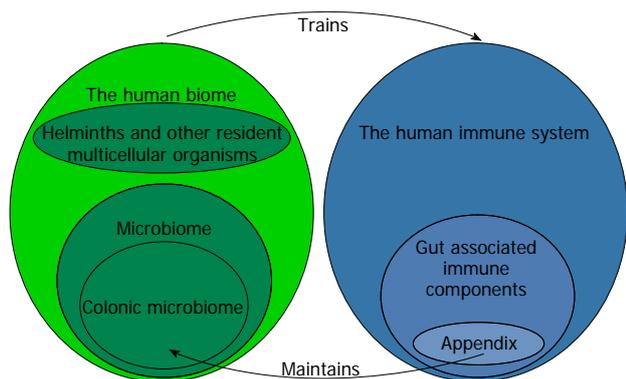
**Core tip:** Although the function of the appendix has remained an enigma for centuries, recently emerging advances in the fields of immunology and gut microbiology have merged with observations made in the clinic to form a coherent picture. Although the appendix is apparently a safe-house for beneficial bacteria, it seems likely that this safe-house does not satisfactorily protect the microbiome from broad spectrum antibiotics. In this view, selection pressures which threatened the microbiome and likely drove the evolution of the appendix have been supplanted in post-industrial society by new threats to the microbiome that the human body is not adapted for.

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### INTRODUCTION

#### *Is appendectomy the removal of a functional organ?*

Appendectomy, like a wide variety of other surgical procedures, is extremely common in industrialized society. However, unlike common surgical procedures that include sterilizations for contraception, Cesarean sections, and inguinal hernia repairs, appendectomies are frequently performed as a prophylaxis for disease. The lifetime risk for appendicitis is only 8.6% for males and 6.7% for females, contrasting to the 12% and 23% lifetime rate of appendectomies performed, respectively<sup>[1]</sup>. These numbers indicate that approximately half of all appendectomies, including more than 60% in females, are



**Figure 1** Interactions between the human biome (left Venn diagram) and the human immune system (right Venn diagram). In this view, all living organisms associated with the human body, either as permanent residents or through transient interactions, are part of the human biome. Two subsets of the biome, helminths and the microbiome, are shown as being part of the human biome. The colonic microbiome, in turn, is shown as being a subset of the microbiome in the Venn diagram of the human biome. Similarly, the appendix is shown as being a subset of the gut associated immune components, which in turn are a subset of the entire human immune system. The idea that the biome “trains” the immune system, equivalent to the view that the immune system is dependent on the biome for proper development, is illustrated. In this model, profound alterations in the biome as a result of post-industrial societies lead to aberrant immune system development, resulting in a variety of immune related pathologies, including appendicitis. The view that the appendix assists in maintaining of the colonic microbiome is also shown. Alterations in the biome that affect immune system training in post-industrial societies predominantly involve compartments of the biome other than the microbiome (*e.g.*, loss of helminths), so the processes leading to appendicitis are generally distinct from processes involved in support of the microbiome by the appendix.

incidental procedures, aimed at averting future episodes of appendicitis. This approach is generally successful, but 36 incidental appendectomies are required to prevent one case of appendicitis<sup>[1]</sup>. Given the large number of appendectomies currently performed, many of them elective, recently emerging evidence regarding the apparent function of the vermiform appendix has justifiably garnered much interest.

The idea that the vermiform appendix is a vestige of evolution was developed more than 150 years ago by Darwin<sup>[2]</sup>. The proposal was simple and made sense in the light of available data: the appendix and small cecum present in humans and some primates is the remainder of a larger cecum used for fermentation in a human ancestor with a diet much higher in fiber<sup>[2]</sup>. However, recent studies using current methods employed in the field that Darwin<sup>[2]</sup> founded have disproved that idea. In summary, a modern cladistics-based approach demonstrates that the appendix has evolved repeatedly in a wide range of animals, that some clades have a propensity to evolve an appendix, and that the evolution of the appendix is usually not associated with a decrease in the size of the cecum. In fact, a recent analysis of 361 mammalian species found a significant direct correlation between appendix and cecum size<sup>[3]</sup>. In other words, the appendix tends to be associated with a large cecum, not a smaller one. At present, many questions regarding the evolution of the appendix remain unanswered: it is not even

known whether the first appendix evolved before or after the first cecum<sup>[4]</sup>, or how often which precedes the other in evolution (given the rise of the appendix more than once during evolution). Although the absolute disproof of Darwin’s views of the appendix is recent, the idea that the appendix is a vestige of evolution has been disputed effectively for more than a century. For example, Berry<sup>[5]</sup> concluded in 1900 that, based on anatomical and phylogenetic data, “The vermiform appendix of man is not, therefore, a vestigial structure. On the contrary, it is a specialized part of the alimentary canal”. Keith<sup>[6]</sup> supported Berry’s views and argued further that that the appendix, rather than being a flawed structure which gives rise to appendicitis, is a victim of changes in the environment due to industrialization: “When we come to realize how slowly evolutionary processes have affected man’s body in past times, we can hardly expect our internal digestive system to adapt itself to the rapid pace demanded by the ever-accumulating resources of civilization”.

When Keith<sup>[6]</sup> recorded his views in 1912, the incidence of appendicitis had profoundly increased in the lifetime of many practicing physicians, and it was therefore correctly surmised that something environmental was causing the disease. The opinion of the day was that changing diet following industrialization was in some way responsible for appendicitis. Although the view that appendicitis was due to an environmental factor or factors in industrial and post-industrial environments was solidified by numerous epidemiologic studies<sup>[7-11]</sup>, it was not until the 1980s that Barker *et al.*<sup>[12-14]</sup> determined that factors associated with indoor plumbing were somehow responsible for appendicitis<sup>[14]</sup>. These intriguing findings by Barker as well as additional work by Strachan on allergic disease<sup>[15]</sup> eventually gave rise to the currently held view that factors within post-industrial culture, including sanitation practices (*e.g.*, toilets and water treatment facilities) and modern medicine, lead to depletion of species normally associated with the ecosystem of the human body, or the “human biome” (not to be confused with the “microbiome”, Figure 1). The resulting state, termed “biome depletion” is associated with a profoundly over-reactive immune system that is prone to a variety of immune related diseases, including appendicitis. Barker’s personal view is that the introduction of running hot water into a home might be the single most telling factor associated with an increased incidence of appendicitis (personal communication to Parker W). Since hot water is necessary for the effective use of soap, and given the effectiveness of soap in biological decontamination, this view makes sense. Here it should be noted that approaches which deal with the consequences of biome depletion are expected to one-day make appendicitis a rare disease. These approaches involve reconstitution of the human biome without abandoning the modern technology, including soap, water treatment facilities and medicine, which so effectively prevents the spread of water-borne disease<sup>[16-18]</sup>.

Despite proof that the appendix is not a vestige of

evolution and that appendicitis is not the result of a faulty structure, the idea that the appendix is a vestige seems attractive simply because removal of the appendix does not, to the practicing physician or to the patients concerned, seem to have deleterious effects. This observation, apparent to everyone, presents a quandary: how can the appendix have some function, but yet appendectomy has no negative side effects? The answer to this quandary is readily apparent if one considers that actual function of the appendix.

In 2003 it was observed that the immune system apparently supports growth of mutualistic biofilms in the mammalian gut<sup>[19]</sup>. This view, although surprising at the time due to prevailing views in the field of immunology, now seems rather obvious in hindsight based on current knowledge regarding microbial ecology and host-microbe relationships<sup>[20-22]</sup>. This new view led to the evaluation of biofilm distribution in the human gut, and biofilms were indeed found to be most abundant in the appendix, where immune tissue had long been known to be the most abundant within the gut. This biofilm distribution in the gut set the stage for a deductive proof regarding the function of the appendix: Since the appendix is a structure harboring microbial biofilms, and since biofilms are protective of bacteria (a long standing observation in the field of microbiology), the appendix is, in essence, a safe house for bacteria (Figure 1). Given the shape and location of the appendix, it would indeed be difficult to imagine how the appendix might not be protective of bacteria.

Given the apparent function of the appendix, it has been proposed that an evolutionary driving force for the emergence of the appendix may be as an aid in the recovery from diarrheal illness associated with gastrointestinal (GI) infection. In this view, fragments of biofilms routinely shed from the appendix would serve as “seeds” for inoculation of the colon with a normal microbial flora following a diarrheal purge<sup>[23]</sup>. This explanation makes sense in light of (1) the relative seclusion of the apex of the appendix from the fecal stream, which presumably affords some protection from pathogenic organisms that might temporarily infect the GI tract; and (2) the pronounced role of diarrheal illness in human survival. Indeed, water-borne diseases followed by dysentery are frequently the leading cause of death during war and natural disasters<sup>[24-27]</sup>, have affected both the rich and the poor<sup>[28]</sup> and are still one of the leading causes of death in developing cultures<sup>[29,30]</sup>. These observations are consistent with the view that that rapid reconstitution of the microbiome and restoration of a normal bowel following diarrheal illness might be adaptive in many circumstances. In fact, the relatively low mortality rate associated with diarrheal illness, less than one percent<sup>[31]</sup>, is possibly a testament to the effectiveness of natural recovery mechanisms such as those that might involve the appendix. Adding further weight to this view, a very recent study by

Guanine *et al*<sup>[32]</sup> found that “the human appendix contains a wealth of microbes, including members of 15 phyla”. Species identified included members of phyla which constitute more than 98% of the normal colonic microbiome (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*), indicating that the appendix possesses a microbial diversity sufficient to reconstitute the microbiome of the colon.

If this inductive rationale is correct, the paradoxical removal of the functional appendix without immediate and substantial harm is readily explained: Although water-borne disease is one of the leading causes of death in developing countries, the use of modern water treatment facilities and sanitation prevents widespread outbreaks of pathogens which might deplete the normal flora from a substantial portion of the population. Further, the absence of starvation and the presence of modern medicine in developed countries minimize the effects of diarrheal illness on the population.

### Causes of appendicitis

Approximately 50% of cases of appendicitis are generally considered to be enigmatic in origin, with the remainder being attributed to a blockage of the appendix. However, work from David Barker during the 1980’s first identified clues which eventually pointed toward the underlying cause of appendicitis. Barker noticed that epidemics of appendicitis followed the introduction of indoor plumbing into various communities. This observation was followed by epidemiologic studies showing that appendicitis is associated with developed but not with developing countries. Almost at the same time, another epidemiologist, Strachan<sup>[15]</sup>, found that a hyper-active immune system is a consequence of the hygienic environment following the industrial revolution<sup>[15]</sup>. Strachan’s observations point toward the idea that appendicitis, like many other allergic, autoimmune, and inflammatory diseases, is a result of biome depletion, a consequence of industrialization<sup>[16-18]</sup>. This culture-related basis for appendicitis explains why the appendix was not selected against during the course of evolution. Many components of the immune system, such as the appendix, are made obsolete by post-industrialized society, and these have also not been selected against during evolutionary history. Not only are these components now obsolete, but these components often become overly sensitive due to an absence of stimulation and cause detrimental health effects, such as ulcerative colitis that is exacerbated by the appendix<sup>[33]</sup>. Another example of a maladapted immune component is the immune compartment that produces immunoglobulin E (IgE). High levels of IgE lead to allergies and other destructive side effects in industrialized societies, but levels significantly higher than those found in industrialized countries are present in developing countries as a result of productive (beneficial) responses to parasitic infections<sup>[34-36]</sup>.

## THE EFFECT OF APPENDECTOMY IN LIGHT OF THE FUNCTION OF THE APPENDIX

Although an appendectomy is a relatively simple surgical procedure, the effects of removing the appendix are not necessarily straightforward. The appendix is associated with the highest concentration of gut associated lymphoid tissue (GALT) in the gut, and the function of the GALT is vastly complex and incompletely understood. Thus, an appendectomy is expected to profoundly alter the immune system with its hundreds or possibly thousands of interconnected components. Numerous functions have been attributed to the GALT, and it remains unknown how appendectomy alters many of those functions. However, some effects are established. First, appendectomy does have a moderating effect on pathogenic inflammatory immune responses of the gut. The observation that patients without an appendix tend to be at less risk for ulcerative colitis is more than 10 years old<sup>[33]</sup>. More recently, Bolin *et al.*<sup>[37]</sup> used appendectomy as a treatment for ulcerative proctitis, a form of colitis, and showed an improvement of symptoms in 90% of patients, with complete remission in 40% of patients<sup>[37]</sup>. Possibly the most straight-forward explanation for this result is that removal of a substantial amount of GALT from the intestinal tract led to decreased immune reactivity in the gut. Whether the “safe-house” function of the appendix had anything to do with the result seems more speculative.

### **The appendix and the initial onset of *Clostridium difficile* colitis**

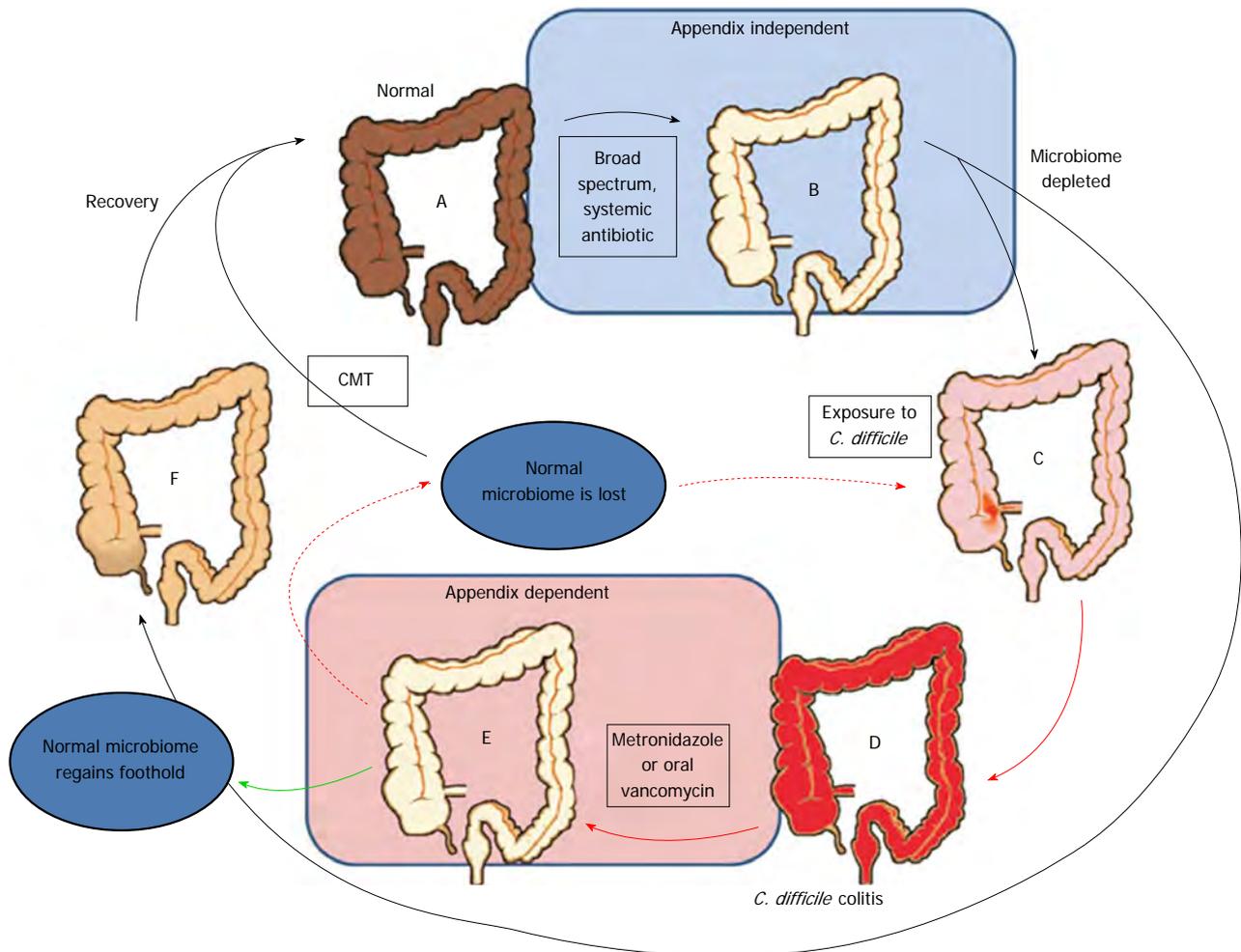
Perhaps the most intriguing effects of appendectomy involve its effects on the incidence of *Clostridium difficile* (*C. difficile*) colitis. *C. difficile* colitis is a pathogenic state associated with overgrowth of the bacterium *C. difficile*, a gram-positive, spore-forming, anaerobic bacillus<sup>[38,39]</sup>, and is generally not seen in individuals with a normal microbiome. However, alteration of the normal flora (generally by antibiotic use) can lead to overgrowth of *C. difficile* and subsequent disease. Recurrent *C. difficile* colitis is not a minor problem in modern medical practice, with one study showing nosocomial *C. difficile* diarrhea present in 3.4 to 8.4 cases per 1000 hospital admissions<sup>[40]</sup>, and an increase in in-hospital mortality from 2.4% to 13.5%<sup>[41]</sup>.

It might at first glance be expected that the appendix, if present, would be protective against *C. difficile* overgrowth. There is, however, at least one central problem with this supposition: it remains unknown if the appendix can effectively protect mutualistic bacteria against the modern antibiotics which generally precede *C. difficile* colitis. It seems reasonable that the appendix has evolved in the presence of enteric pathogens and thus that it may be effective in helping the body to recover from infectious disease. However, the use of high dose antibiotics is a very recent development in human history, and thus it is not reasonable to assume that the appendix may be

protective under these conditions. To our knowledge, no studies have addressed this issue. Our laboratory has assessed the protection from antibiotics afforded by immune-mediated biofilms *in vitro*, and found that immune mediated biofilms formed by one species (*Escherichia coli*) are poorly protected from antibiotics. However, much additional work needs to be done in this field using a wide range of microbial species as well as whole animal models before any sort of answer which might have clinical implications can be obtained.

The supposition that the appendix, if indeed it is a safe-house for bacteria, should be protective against *C. difficile* colitis has a second potential flaw: If indeed the appendix does not protect mutualistic bacteria from antibiotic use, the appendix could hypothetically protect those organisms which are resistant to antibiotics, such as *C. difficile*, from a diarrheal purge. Thus, if the appendix performs its function perfectly, it could hypothetically increase the incidence of *C. difficile* colitis in the face of antibiotic use. Fortunately, this does not appear to be the case. At present, clinical data point toward the idea that the presence or absence of an appendix does not strongly affect the propensity for the initial onset of *C. difficile* colitis. In a study by Im *et al.*<sup>[42]</sup>, 80% of their patients with *C. difficile* colitis (203 out of 253) had an appendix, which is only slightly lower than the percentage found in the total population<sup>[1]</sup>. Another study, by Merchant *et al.*<sup>[41]</sup>, obtained essentially identical results, with 80% of their patients with *C. difficile* colitis (109 out of 136) having an appendix. Merchant *et al.*<sup>[41]</sup> found that 82% of “normal” individuals (in their study, patients without GI complaints) had an appendix, as would be expected based on larger studies<sup>[1]</sup>. However, these observations do not directly address the actual effect of the appendix on the propensity for *C. difficile* colitis following antibiotic use, since they do not address the effect of appendectomy on the use of antibiotics. In other words, the data indicate that appendectomy does not affect the risk for *C. difficile* colitis, but it does not indicate whether an appendectomy might affect the risk for *C. difficile* colitis following antibiotic treatment. Since Merchant *et al.*<sup>[41]</sup> did not control for antibiotic treatment, increased antibiotic use in those with an appendix, if it exists, would have confounded the study. Nevertheless, the observations do clearly indicate that the loss of an appendix is not associated with a dramatically increased risk for an initial onset of *C. difficile* colitis.

As stated above, it is possible that a perfectly functional appendix, if indeed it did not protect the normal flora from antibiotics, might selectively protect antibiotic resistant organisms such as *C. difficile* from a diarrheal purge. This possibility has been previously proposed by Merchant *et al.*<sup>[41]</sup>. However, since the relative number of patients with and without an appendix in patient groups with *C. difficile* colitis is essentially the same as that in the normal population, the possibility that the appendix preferentially protects *C. difficile* seems extremely unlikely. Further, appendectomy itself affords a much lower risk of *C. difficile* colitis (0.2%) compared to colectomy (1.11%)



**Figure 2** The cycle of microbiome depletion with antibiotics, the occurrence of *Clostridium difficile* colitis, and recovery of the microbiome. The cycle is initiated when the normal colon (A) is depleted of its microbiota using broad spectrum antibiotics (B). Although the microbiota often recovers spontaneously from such treatment, the patient is at risk of *Clostridium difficile* (*C. difficile*) colitis (C and D) in a fashion that is independent of the presence of an appendix. Although *C. difficile* colitis is often effectively treated with metronidazole or vancomycin (E), the microbiome can fail to normalize, leading to recurrent *C. difficile* colitis. This cycle of treatment followed by recurrence is indicated by the red arrows. The presence of a vermiform appendix enhances recovery (A and F) of a normal microbiome following *C. difficile* colitis (green arrow), thus averting the cycle of recurrent *C. difficile* colitis. Colonic microbiota transplants (CMT) are also effective at restoring the normal flora and interrupting the cycle of recurrent *C. difficile* colitis.

small-bowel resection (1.17%) and gastric resection (1.02%)<sup>[41]</sup>, further suggesting that the appendix may be relatively uninvolved in the initial onset of *C. difficile* colitis. In addition, the fact that an intact appendix protects against recurrent (as opposed to the initial onset of) *C. difficile* colitis (see below) argues strongly against this view. However, again, it is not known to what extent the presence or absence of an appendix might affect antibiotic use, the major trigger for *C. difficile* colitis. This factor probably needs to be examined before any firm conclusions can be drawn.

**The appendix and recurrent *C. difficile* colitis**

Strong evidence from Im *et al*<sup>[42]</sup> study indicates that the appendix may play a protective role in recurrent *C. difficile* colitis. Im *et al* found a 2.5-fold increased risk of recurrent *C. difficile* colitis in patients without an appendix compared to those with an appendix. Figure 2 illustrates a possible scenario that potentially explains the connection

between the appendix, the initial onset of *C. difficile* colitis, and recurrent *C. difficile* colitis. The central issue revolves around the use of broad spectrum antibiotics which initiate the initial *C. difficile* colitis, and the more limited antibiotic treatments used after the first *C. difficile* infection. The standard of care for recurrent and severe *C. difficile* colitis is oral vancomycin, a treatment that is limited to the lumen of the bowel. Given the position of the appendix out of the main flow of the bowel, it seems likely that it may indeed be effective at protecting the normal flora from oral vancomycin, just as it putatively protects the normal flora from contamination by pathogens in the main fecal stream.

Consistent with the idea that the connection between recurrent *C. difficile* colitis and the appendix involves the bacterial safe-house function of the appendix, recurrent *C. difficile* colitis can be rapidly resolved using fecal microbiome transplants<sup>[43-45]</sup>. This observation indicates that *C. difficile* colitis is indeed an issue involving a depleted gut

microbiome, thus adding support to the idea that an appendix might help restore the gut microbiome in times of stress. Indeed, proof of a depleted biome in recurrent *C. difficile* colitis patients has been provided by phylogenetic analyses of stool samples in patients with recurrent *C. difficile* colitis: decreased bacterial diversity<sup>[46]</sup> as well as a deficiency of Firmicutes and Bacteroidetes<sup>[47]</sup> have been demonstrated in those patients.

Although the function of the appendix as a safe-house for the colonic microbiome explains the clinical observations illustrated in Figure 2, an alternative, although not mutually exclusive, explanation also exists: as noted above, appendectomy probably lowers the immunoreactivity of the gut, and thus may lower the ability of the gut to respond to *C. difficile*. Thus, the loss of the appendix may, hypothetically, reduce the ability of the immune system to mount an immune response to *C. difficile*, which is known to be important in the resolution of the colitis. Thus, a second explanation for the connection between appendectomy and recurrent *C. difficile* colitis shown in Figure 2 may be that the immunosuppressive effect of appendectomy impedes the immune response to *C. difficile*, thus putting the patient at risk for recurrent *C. difficile* colitis. Consistent with this view, Im's data also indicated that increasing age (> 60 years), which is associated with reduced immune function, was also a risk factor for recurrent *C. difficile* colitis<sup>[42]</sup>. In this view, the lack of a connection between the initial onset of *C. difficile* colitis and appendectomy may be due to the lack of time necessary to mount an immune response that would be dependent on the immune tissue of the appendix.

### **The appendix and gastrointestinal pathology unrelated to *Clostridium difficile***

A potentially alarming observation was made in the study by Merchant *et al*<sup>[41]</sup>: 31 percent (39 out of 121) of their patients which were tested for *C. difficile* colitis but which were found negative for *C. difficile* colitis had a previous appendectomy. This number is very significantly greater than is expected if the presence or absence of an appendix was not related in some way: The probability (binomial test) of observing 38 out of 121 patients with an appendectomy is < 0.0001 given a null hypothesis of 0.18 (a population-wide rate of 18% appendectomy). If this observation is confirmed by additional studies, it would indicate an association between appendectomy and complications which resemble *C. difficile* colitis (and thus induce clinicians to order a test for *C. difficile*), but which are in fact not associated with *C. difficile*. This idea deserves further attention before any firm conclusions can be drawn, but the observations made by Merchant *et al*<sup>[41]</sup> nevertheless have great potential importance, and certainly raise a sense of urgency for further study of this topic.

The strongest connection between appendectomy and inflammatory diseases unrelated to *C. difficile* colitis of the bowel is provided by the Merchant *et al* study<sup>[41]</sup>. However, additional indirect evidence for this connection

is provided by the effectiveness of colonic microbiota transplants in treating some patients whose disease has resisted other therapeutic options<sup>[43,44]</sup>. The effectiveness of microbiota transplants in some patients strongly indicates that a loss of the normal microbiome is at the root of the symptoms experienced by these patients. Thus, to the extent that the appendix assists in maintenance of the microbiome, the lack of an appendix may influence the incidence of these idiopathic cases. At the same time, it is recognized that loss of the microbiome by a wide range of modern medical interventions (*e.g.*, sterile birth practices, broad spectrum antibiotics) may circumvent any protective role of the appendix, and direct assessment of the rate of appendectomy in patients with an altered microbiome should be undertaken.

### **Alternatives to appendectomy**

Acute appendicitis is the widely recognized indication for appendectomy, although alternatives involving medical treatment are being considered. Medical treatment alone has the substantial disadvantages that (1) heavy use of antibiotics must be employed, which is not without its own side effects; and (2) recurrence of appendicitis following antibiotic use is possible. A controlled study by Eriksson *et al*<sup>[48]</sup> compared the outcomes of patients treated with a 10 d antibiotic regimen (cefotaxime and tinidazole in the hospital for two days followed by eight days of oral antibiotics) versus patients who underwent appendectomy. They found that patients on the antibiotic regimen used significantly less morphine, had lower white blood cell counts, and had less pain at follow up. Two surgical patients underwent post-operative antibiotic therapy for complications, and there was an appendicitis recurrence rate of 35% in the antibiotic group. Another study by Styruud *et al*<sup>[49]</sup> saw an 86% success rate with antibiotics with only a 14% recurrence rate within one year. The complication rate in the surgical group was 14%. These studies suggest that acute non-perforated appendicitis can be treated conservatively with an antibiotic regimen; however, the risk of recurrence should be compared to the risk of surgical complication in the patient.

Antibiotics have also proven effective at delaying appendectomy. Nine sailors who were diagnosed with appendicitis while serving at sea received various antibiotic regimens until the men could be taken to a hospital, and all achieved positive outcomes<sup>[50]</sup>. A study of 695 children showed that an antibiotic regimen in children allowed the appendectomy to be delayed up to 18 h after admission without an increase in complications<sup>[51]</sup>.

## **CONCLUSION**

It seems highly likely that the appendix, evolved in a time before sewer systems and water treatment facilities existed, is somewhat out of place in post-industrial society. Removal of the appendix and its associated GALT does afford some degree of immune suppression, which can be advantageous in a post-industrial environ-

ment rampant with inflammatory diseases of the bowel. However, removal of the appendix may also impede the ability of the body to replenish helpful bacteria, and/or appendectomy might hinder helpful immune responses, such as those directed at *C. difficile*. Whatever the cause, appendectomy appears to be associated with an increased risk for recurrent *C. difficile* colitis, which is not a minor problem in modern medical practice. Indeed, one study found nosocomial *C. difficile* diarrhea present in 3.4 to 8.4 cases per 1000 hospital admissions<sup>[40]</sup>, and an increase in in-hospital mortality from 2.4% to 13.5%<sup>[41]</sup>. With this in mind, further studies aimed at biome reconstitution, which are predicted to eliminate the vast majority of appendicitis cases, and thus the need for most appendectomies, are warranted. Further, studies regarding the long term effects of incidental appendectomies should be carefully considered.

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## MicroRNA-21 as a potential colon and rectal cancer biomarker

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ing future prospects.

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**Key words:** Colorectal cancer; MicroRNA; Diagnosis; Treatment; Chemotherapy

**Core tip:** We summarize the latest study findings about microRNA-21 in colorectal cancer through a systematic review of literature. We recommend microRNA-21 as one of the most important microRNAs, which is rapidly emerging as a novel biomarker, with good potential as a diagnostic and therapeutic target.

### Abstract

Colorectal cancer (CRC) is one of the most common malignant diseases worldwide and the prognosis is still poor although much progress has been achieved in recent years. In order to reduce CRC-related deaths, many studies are aimed at identifying novel screening- and prognosis-related biomarkers. MicroRNAs (miRNAs) are a class of 18-27-nucleotide single-stranded RNA molecules that regulate gene expression at the post-transcriptional level. It has been demonstrated that miRNAs regulate a variety of physiological functions, including development, cell differentiation, proliferation, and apoptosis. They play important roles in various physiologic and developmental processes and in the initiation and progression of various human cancers. It has been shown that miRNAs can critically regulate tumor cell gene expression, and evidence suggests that they may function as both oncogenes and tumor suppressor genes. In CRC, miRNAs-21 is one of the most important miRNAs and is rapidly emerging as a novel biomarker in CRC, with good potential as a diagnostic and therapeutic target. In this review, we summarize the latest research findings of the clinicopathological relevance of miRNAs-21 in CRC initiation, development, and progress, highlighting its potential diagnostic, prognostic, and therapeutic application, as well as discuss-

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### INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of death in the United States. It was estimated that there were about 142570 new cases diagnosed and 51370 deaths in 2010<sup>[1]</sup>. Progress in diagnosis and treatment has had a positive effect in improving overall survival, with more patients being diagnosed in the early stage of the disease, but the outcomes of patients diagnosed with advanced stage disease remains quite poor<sup>[2]</sup>. Long-term survival and better prognosis of patients depend on the stage of the tumor at the time of detection. Fecal occult blood testing and tumor markers (*e.g.*, carcinoembryonic antigen) are used as the primary screening tools, with colonoscopy reserved for patients testing positive. However, they are generally considered to lack the desired convenience, sensitivity and specificity<sup>[3]</sup>. There are currently no tests or biomark-

ers that precisely predict the presence of early tumors, recurrence, sensitivity to chemotherapy and long-term survival. It is clear that improvements in early detection of primary and recurrent disease are required.

MicroRNAs (miRNAs) are a family of small, non-coding RNAs (19-22 nucleotides) which post-transcriptionally regulate gene expression. In general, miRNAs are transcribed as a group called the pri-miRNA complex, which is cleaved in the nucleus to form the pre-miRNA which is then translocated to the cytoplasm where they undergo final maturation into a functional miRNA<sup>[4]</sup>. Once in the cytoplasm, the miRNAs regulate gene expression by binding to the 5'-untranslated region of their target mRNA resulting in degradation of the double-stranded mRNA mediated by the Dicer complex. More than 700 miRNAs have thus far been identified in plants, viruses, animals and humans, and this number continues to increase (www.mirbase.org). Studies have shown that about 30% of human genes are regulated by miRNAs<sup>[5]</sup>. This wide regulation has implications in many important cellular functions including development, differentiation, proliferation, and programmed cell death<sup>[6-8]</sup>. Given the critical regulatory roles miRNAs serve, it is no surprise that they have been shown to be associated with many cancers<sup>[9]</sup>. CRC is a complex genetic disease characterized by uncontrolled proliferation, migration, invasion, and failure of apoptotic cell death, due to oncogene activation and tumor suppressor gene defects<sup>[10]</sup>. Many miRNAs which mediate cell growth and tumor progression have been found to be upregulated in CRC including miR-20, miR-21, miR-17-5p, miR-15b, miR-181b, miR-191 and miR-200c<sup>[9,11-14]</sup>. While lower levels of mature miRNAs such as miR-34a, miR-126, miR-143, miR-145 and miR-342 are also found, suggesting that they act as tumor suppressor miRNAs<sup>[15-18]</sup>. This deregulation of various miRNAs has been associated with tumor diagnosis and prognosis indicating that they might be potential biomarker in clinical application<sup>[3,19-21]</sup>. Multiple studies have identified that miR-21 plays a significant role in cancer biology, diagnostics and prognosis. In this article, we review the literature demonstrating the importance of miR-21 in CRC, summarize the association of miR-21 expression level with CRC diagnosis and prognosis, and discuss the potential therapeutic implications for the future.

## MIR21 IN COLORECTAL CANCER

Human miR-21 (hsa-miR-21) was cloned from HeLa cell total RNA and is highly conserved among species including human, rat, mouse, fish and frog<sup>[22]</sup>. It is located on chromosome 17q23-1 overlapping with the TMEM49 gene, a human homologue of rat vacuole membrane protein-1. MiR-21 encodes a single hairpin and is regulated by its own promoter containing binding sites for AP-1 and PU.1 transcription factors<sup>[23]</sup>. Experimental data has shown that miR-21 functions in many cell types as an anti-apoptotic and pro-survival factor and plays a significant role in cancer biology and prognosis<sup>[24-26]</sup>. Asangani *et al.*<sup>[26]</sup> transfected Colo206f cells with miR-21 and found

**Table 1 Current screening methods and guidelines for colorectal cancer**

Method	Sensitivity	Interval	Society
Fecal tests			
FOBT		Yearly	USPSTF, ASGE, USMSTF
FIT	65.8% <sup>[32,33]</sup>	Yearly	
Fecal DNA	50%-60% <sup>[34]</sup>	Unspecified	USMSTF
Serum markers			
CEA	30% <sup>[35]</sup>		
CA19-9			
Imaging tests			
DCBE	85%-97% <sup>[36]</sup>	Every 5 years	USMSTF
CTC	55%-94% <sup>[37]</sup>	Every 5 years	USMSTF
Optical tests			
FS		Every 5 years Every 10 years	USPSTF, ASGE, USMSTF
FC			USPSTF, ASGE, USMSTF

FOBT: Fecal occult blood test; FIT: Fecal immunochemical based stool tests; CEA: Carcinoembryonic antigen; DCBE: Double-contrast barium enema; CTC: Computed tomography colonography; FS: Flexible sigmoidoscopy; FC: Flexible colonoscopy; USPSTF: United States Preventive Services Task Force; ASGE: American Society for Gastrointestinal Endoscopy; USMSTF: Multi-Society Task Force on Colorectal Cancer.

significant suppression of PDCD4 proteins *in vitro*. Resected normal and tumor tissues of 22 CRC patients demonstrated that miR-21 expression has a direct correlation with tumor invasion and metastasis.

### miR-21 in adenomas

It is clear that the majority of CRCs begin as benign adenomas, and through a series of accumulated genetic events, end up as invasive tumors. However, not all polyps will progress to invasive carcinomas. In fact, it is estimated that up to 20% of benign, subcentimeter adenomas will ultimately regress<sup>[27,28]</sup>. Therefore, it seems that the key to preventing polyps from progressing to malignant carcinomas is being able to determine which ones have the potential to progress and removing them at the benign stage. Interestingly, increased expression of several miRNAs such as miR-21, miR-31, miR-96, miR-221, miR-191, miR-19a, and miR-135b has been shown to correlate with the presence of adenomas<sup>[29,30]</sup>. In fact, Yamamichi *et al.*<sup>[31]</sup> analyzed miR-21 expression patterns in different stages of CRC development using *in situ* hybridization, and found higher miR-21 expression in precancerous adenomas but not in non tumorigenic polyps. Furthermore, the frequency and extent of miR-21 expression increased during the transition from precancerous colorectal adenoma to advanced carcinoma. This demonstrates that expression of miR21 in benign colon adenomas may represent an early event in the progression to carcinoma.

### Expression of miR-21 as a screening test for colorectal cancer

Current recommendations for CRC screening are found in Table 1<sup>[32-37]</sup>. Fecal occult blood testing is a widely used test but its low specificity and sensitivity limits its clinical

use, particularly for early detection. Newer screening tests are taking advantage of the presence of stem cells from human exfoliated deciduous teeth cells in the stool and are using various molecular tests to examine these cells for genetic events consistent with malignant changes. Expression levels of miRNAs offer attractive new potential biomarkers as they are uniquely stable and may represent some of the earliest changes in adenomas. Ng *et al.*<sup>[38]</sup> reported high expression levels of miRNAs in colorectal tumors and plasma. Of the panel of 95 miRNAs analyzed by real-time polymerase chain reaction (PCR), five were upregulated in both plasma and tissue. The results were again validated using the plasma of 25 patients with CRC and 20 healthy controls. In these studies, the miRNAs 21, 17-3p, and 92 were elevated in patients with CRC ( $P < 0.0005$ ). The authors further demonstrated that the plasma levels of these markers were significantly reduced after surgery in 10 patients with CRC ( $P < 0.05$ ) suggesting that the high levels specifically indicate the presence of a carcinoma. Kristina *et al.* tested the levels of 15 miRNAs in stool and colorectal tissue samples from 15 patients with CRC and five healthy individuals<sup>[39]</sup>. Although, variability was more pronounced among the stool samples than the tissue samples, the authors concluded that specific miRNA expression profiles could be defined, suggesting that stool is yet another biological material in which miRNAs are preserved and are amenable for early diagnosis of CRC. A stage-independent, sensitive, and specific marker for CRC in plasma or stool would be clinically important, and clearly these promising results support further assessment of miRNAs as potential biomarkers both in adenoma and extracellular fluids.

### **miR-21 expression levels and prognosis**

The prognosis of patients with CRC is associated with tumor stage and phenotypic characteristics of resected cancer specimens such as tumor grade, positive lymph nodes, and angiolymphatic invasion<sup>[40]</sup>. Unfortunately, recently identified genomic and proteomic biomarkers, tumor cell mutations, and microsatellite instability cannot be recommended for routine clinical use because of insufficiently available data<sup>[41]</sup>. However, markers of prognosis are needed to help stratify patients into high risk thereby identifying patients who are likely to benefit from further therapy. Many studies on tumor biomarkers have been undertaken<sup>[42]</sup>. However, no study has identified a new marker that has been validated in clinical trials. The miRNAs represent particularly attractive markers as they seem to be micromanagers of cellular gene expression and may represent the earliest events responsible for carcinogenesis. In fact, studies have shown that the expression levels of different miRNAs, such as miR-21, miR-320, miR-498, miR-106a and miR-200c, correlate with disease-free and overall survival<sup>[43]</sup>.

miR-21 may be a particularly attractive target as it has been shown to regulate the expression of many genes thought to be important in carcinogenesis. Target validation studies on putative miR-21 targets in breast cancer samples and CRC cells have demonstrated a link between

miR-21 expression levels and the p53 tumor suppressor, and also demonstrated that the tumor suppressors PDCD4 and maspin are targets of miR-21<sup>[44]</sup>. Consistent with the importance in the process of carcinogenesis, Staby *et al.*<sup>[45]</sup> demonstrated that higher miR-21 expression levels were correlated with advanced cancer stages, worse outcome, poor response to therapy, and shorter disease-free survival. Additionally, they found that miR-21 levels were positively correlated with cancer stage, lymph node involvement, and development of distant metastasis.

The most comprehensive analysis of miRNA expression in CRC performed to date tested two cohorts of 197 colon cancer patients by utilizing microarrays containing 389 miRNAs probes<sup>[46]</sup>. This analysis revealed 37 miRNAs which were differentially expressed in stage II colonic adenocarcinoma compared with adjacent normal tissue using a test set and two validation cohorts. In one of the cohorts, miR-20a, miR-21, miR-106a, miR-181b, and miR-203 were found to be overexpressed in tumor tissues with high tumor to normal ratios, as well as being associated with poor overall survival. However, the prognostic relevance could be confirmed in the validation set for only one of the candidates, miR-21, and the clinical and biological implications of differential expression of the remaining miRNAs were unclear. Similar conclusions were drawn from a study of 29 tumor samples, in which miR-21 expression was associated with poor survival and therapeutic outcome in stage II and III CRC<sup>[46]</sup>. Nielsen *et al.*<sup>[47]</sup> reported the expression of miRNA-21 in 130 colon and 67 rectal stage II cancer specimens using high-affinity locked nucleic acid (LNA) probes. High levels of miR-21 correlated with shorter disease-free survival (hazard ratio: 1.28; 95% confidence interval: 1.06-1.55;  $P = 0.004$ ) in the stage II colon cancer patient group, whereas no significant correlation with disease-free survival was observed in the stage II rectal cancer group.

### **miR-21 expression and implications for treatment**

**miR-21 and response to chemotherapy:** The current treatment for CRC involves a multidisciplinary approach including surgery supplemented with chemotherapy and radiation therapy in certain instances. In general, patients with node-positive disease benefit from chemotherapy. However, there may be a subgroup of patients with node-positive disease who are at low risk of recurrence, as nearly 40% of patients randomized to a no-treatment arm in chemotherapy trials did not develop a recurrence<sup>[48]</sup>. In addition, it is clear that some patients with node-negative disease who have advanced T-stage tumors are at high risk of developing recurrences<sup>[49]</sup>. A test that would allow for the accurate stratification of patients with stage II and III disease into low and high risk would be very clinically useful. Recently, the role of miRNAs in predicting the response to 5-fluorouracil (5-FU)-based chemotherapy in CRC treatment has been explored. A significant focus has been placed on the value of miR-21 expression levels and their abilities to predict both response to and need for chemotherapy<sup>[50]</sup>.

Rossi *et al.*<sup>[51]</sup> utilized two subclones from the human

**Table 2** MicroRNA-21 expression in colorectal cancer

Ref.	Regulation	Biological material tested	Detection method	Clinical relevance	Comment
Tumor development Fassan <i>et al</i> <sup>[60]</sup>	Up	300 polypoid lesions of the colon mucosa	RT-PCR ISH	Significant miR-21 upregulation in preneoplastic/neoplastic samples	High miR-21 expression is consistent with PDCD4 downregulation
Yantiss <i>et al</i> <sup>[61]</sup>	Up	24 patients < 40 years 45 patients ≥ 40 years	RT-PCR	Significantly higher expression	
Tumor diagnosis Link <i>et al</i> <sup>[62]</sup>	Up	Stool samples	RT-PCR	Higher expression in patients with adenomas and CRCs	May be an excellent candidate of a noninvasive screening test for colorectal neoplasms
Tumor prognosis Chang <i>et al</i> <sup>[63]</sup>	Up	48 colorectal tumors, 61 normal tissues, 7 polyps	RT-PCR	Disease recurrence	miR-21 post-transcriptionally modulates PDCD4 <i>via</i> mRNA degradation
Nielsen <i>et al</i> <sup>[47]</sup>	Up	130 stage II colon and 67 stage II rectal cancer specimens	ISH	Shorter disease-free survival in colon cancer, but not in rectal cancer	
Kulda <i>et al</i> <sup>[64]</sup>	Up	46 paired tissue samples 30 tissue samples with live metastasis	RT-PCR	Disease-free interval	
Schetter <i>et al</i> <sup>[46]</sup>	Up	196 paired tissues	RT-PCR	Association with cancer-specific mortality, including stage II patients alone	miR-21 expression are independent predictors of colon cancer prognosis and may provide a clinically useful tool to identify high-risk patients
Schetter <i>et al</i> <sup>[46]</sup>	Up	US cohort: 84 patients; Hong Kong cohort: 113 patients	MicroRNA microarray, RT-PCR	Poor survival and poor therapeutic outcome	

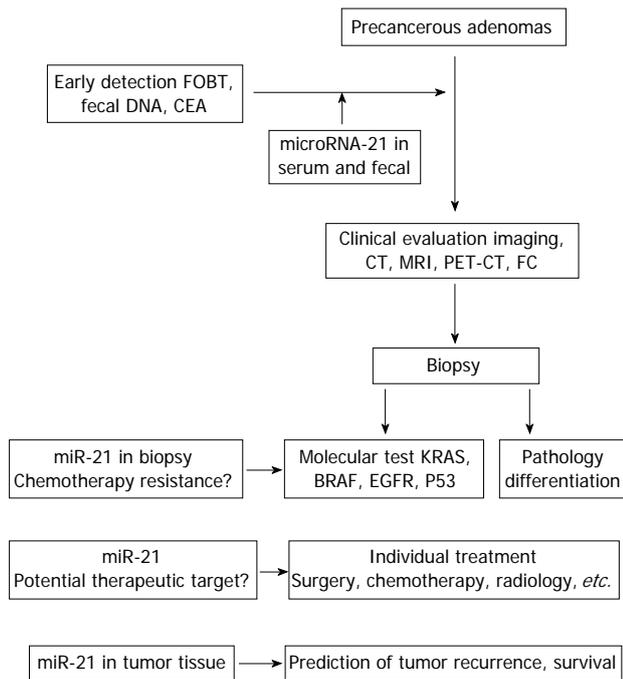
RT-PCR: Reverse transcription-polymerase chain reaction; ISH: *In situ* hybridization; PDCD4: Programmed cell death protein 4.

CRC cell lines HT29 and HCT116 to investigate the effect of 5-FU on miRNA expression and also to determine patterns of expression that correlated with response to therapy. Quantitative real-time PCR revealed that 5-FU upregulated 19 and downregulated three miRNAs. While some changes in miRNA expression were consistent with the antitumor effects of the drug, others were not, such as upregulation of miR-21 and the polycistronic miR-17-92 cluster (which include miR-19a and miR-20). In fact, a number of miRNAs that are already overexpressed in neoplastic tissues, including miR-21, have been shown to be upregulated in colon cancer cell lines treated with 5-FU<sup>[51]</sup>. Tomimaru *et al*<sup>[52]</sup> found that hepatocellular carcinoma cells transfected with pre-miR-21 were significantly resistant to 5-FU, while the 5-FU sensitivity of transfected anti-miR-21 was weakened by transfection with siRNAs of the target molecules, PTEN and PDCD4. This finding may be a cell-specific defense mechanism to survive 5-FU treatment. Svoboda *et al*<sup>[53]</sup> found significant changes in miRNA expression in 35 patients with rectal carcinoma undergoing preoperative capecitabine chemoradiation therapy. Tumor biopsies were taken before starting therapy and after 2 wk of therapy. The extent of the tumor response to the therapy was investigated microscopically by an experienced pathologist according to Mandard's tumor regression criteria. In addition, the levels of miRNAs were evaluated using real-time PCR. The authors found dramatic changes in the expression levels of many miRNAs including miR-21,

miR-10a, miR-145, miR-212, miR-339, miR-361. However, only two miRNAs, miR-125b and miR-137, were found to be significantly increased after 2 wk of therapy and these miRNA expression levels had a positive correlation with a poorer tumor regression response<sup>[53]</sup>. These types of studies highlight the potential for using miRNA expression profiles to predict the response to chemotherapy. However, verification of the targets in adequately designed clinical panels is the important next step that has yet to be taken.

**miR-21 maybe a potential therapeutic target in colorectal cancer treatment:** miRNAs are important regulators of gene expression and may present potentially interesting therapeutic targets in cancer. The synthesis, maturation and activity of miRNAs can be manipulated with various oligonucleotides that encode the sequences complementary to mature miRNAs. By influencing particular miRNAs, a cascade of pathways could be modified to inhibit tumor growth.

Wong *et al*<sup>[54]</sup> reported the application of 20-O-methyl-and/or DNA/LNA-mixed oligonucleotides to specifically inhibit miR-21 in cultured glioblastoma and breast cancer cells suppressed cell growth *in vitro* in association with increased caspase-mediated apoptosis<sup>[55]</sup>. Suppression of miR-21 also significantly reduced invasion and lung metastasis in MDA-MB-231 metastatic breast cancer<sup>[56]</sup>. Although there are at present no clinical reports describing therapy targeting miR-21 in CRC treatment,



**Figure 1** miR-21 as a potential biomarker in colon and rectal cancer. CT: Computed tomography; MRI: Magnetic resonance imaging; PET-CT: Positron emission tomography-computed tomography; FC: Fiber colonoscopy; FOBT: Fecal occult blood test; CEA: Carcinoembryonic antigen.

most seem to have optimistic views on the future utility of miR-21 as therapeutic targets but further studies are clearly needed<sup>[57-59]</sup>. Expression of microRNA-21 and the clinical relevance in CRC are summarized in Table 2.

## FUTURE PERSPECTIVES

The role of miRNAs in CRC presents potentially exciting new opportunities for future investigations to determine the use of miRNAs as potential biomarkers for prognosis at the time of diagnosis as well as to determine their ability to predict the response to chemotherapy. In addition, the potential for miRNAs to serve as targets for new chemotherapeutic treatments has yet to be realized. Among the many miRNAs that have been associated with clinical outcomes, miR-21 has been consistently shown to be dysregulated in CRC. Many of the early studies relating miR-21 to CRC have been performed *in vitro* on established cell lines. In addition, studies using human tumor tissue have been performed in a retrospective fashion, which limits the conclusions because of inherent study bias. If these micromanagers of cell processes are to be useful tools in the diagnosis or treatment of colon cancer, we will have to study them in well-designed prospective trials. It remains to be discovered if these types of molecular expression profiles will be used in clinical practice (Figure 1).

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## Doxorubicin-eluting bead vs conventional transcatheter arterial chemoembolization for hepatocellular carcinoma before liver transplantation

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### Abstract

**AIM:** To assess the possible effect of two different types of preoperative transcatheter arterial chemoembolization (TACE) on recurrence-free survival after liver transplantation (LT) in patients with hepatocellular carcinoma (HCC) and to analyze the effects of TACE on tumor histology.

**METHODS:** We retrospectively analyzed the histological features of 130 HCC nodules in 63 native livers removed at transplantation. Patients who received any other type of treatment such as radiofrequency tumor ablation, percutaneous ethanol ablation or who were not treated at all were excluded. All patients in the present study were within the Milan Criteria at the last imaging findings before transplantation. Doxorubicin-eluting bead TACE (DEB-TACE) was performed in 22 patients (38 nodules), and conventional TACE (c-TACE) in 16 (25 nodules). Patients' and tumors' characteristics were retrospectively reviewed. We performed a per-nodule analysis of the explanted livers to establish the mean percentage of necrosis of any nodule treated by TACE (conventional or DEB) and a per-patient analysis to establish the percentage of necrosis in the cumulative tumor area, including 21 nodules not reached by TACE. Inflammatory and fibrotic changes in the tissue surrounding the tumor nodule were analyzed and categorized as poor/absent, moderate and enhanced reaction. Uni- and multivariate analysis of risk factors for HCC-recurrence were performed.

**RESULTS:** The number and diameter of the nodules, the time spent on the waiting list and the number of treatments were similar in the two groups. A trend towards higher appropriate response rates (necrosis  $\geq$

90%) was observed in the DEB-TACE group (44.7% vs 32.0%,  $P = 0.2834$ ). The mean percentage of necrosis in the cumulative tumor area was  $58.8\% \pm 36.6\%$  in the DEB-TACE group and  $50.2\% \pm 38.1\%$  in the c-TACE group ( $P = 0.4856$ ). Fibrotic and inflammatory reactions surrounding the tumor nodule were markedly more common in the DEB-TACE group ( $P < 0.0001$ , for both the parameters). The three-year recurrence-free survival was higher in DEB-TACE-treated patients than in conventionally treated patients (87.4% vs 61.5%,  $P = 0.0493$ ). Other factors affecting recurrence-free survival included viable tumor beyond Milan Criteria on histopathological examination, the percentage of necrosis on CTA  $\leq 50\%$  and a pre-transplant serum  $\alpha$ -fetoprotein level greater than 70 ng/mL. On multivariate analysis, the lack of treatment with DEB-TACE, high levels of  $\alpha$ -fetoprotein and viable tumor beyond Milan Criteria at histology examination were identified as independent predictors of tumor recurrence.

**CONCLUSION:** DEB-TACE can effectively promote tumor necrosis and improves recurrence-free survival after LT in HCC.

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**Key words:** Liver transplantation; Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Doxorubicin-eluting bead; Tumor histology; Recurrence-free survival; Locoregional therapies

**Core tip:** The manuscript reports the experience with a newer technique of transcatheter arterial chemoembolization (TACE) that uses doxorubicin-eluting beads (DEB) for the treatment of hepatocellular carcinoma in liver transplant candidates. The results of DEB-TACE were compared to those of conventional TACE, and remarkably, a significantly higher recurrence-free survival after liver transplantation was observed in patients who were treated with DEB-TACE. The histological pattern observed in the area surrounding the tumor nodules of DEB-TACE patients was characterized by an intense inflammatory and fibrotic reaction, which could play a role in limiting tumor spread during waiting list time.

Nicolini D, Svegliati-Baroni G, Candelari R, Mincarelli C, Mandolesi A, Bearzi I, Mocchegiani F, Vecchi A, Montalti R, Benedetti A, Risaliti A, Vivarelli M. Doxorubicin-eluting bead vs conventional transcatheter arterial chemoembolization for hepatocellular carcinoma before liver transplantation. *World J Gastroenterol* 2013; 19(34): 5622-5632 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5622.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5622>

## INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for approximately 5% of all cancers, with more than 500000 new cases diagnosed each year<sup>[1]</sup>. The link between liver cir-

rhosis and HCC is well known; more than 90% of HCCs develop in cirrhotic livers, and 3%-8% of cirrhotic patients are diagnosed as HCC carriers each year<sup>[2]</sup>. Ideally, liver transplantation (LT) is the best treatment option for HCC, as it removes both the tumor and the underlying chronic condition<sup>[3]</sup>.

Milan Criteria (MC) of LT candidates with HCC, based on the number and size of the tumor nodules, has led to 5-year survival rates well above 70% and recurrence rates below 15%<sup>[4]</sup>. Currently, more than 30% of LT recipients in the United States are HCC carriers<sup>[5]</sup>.

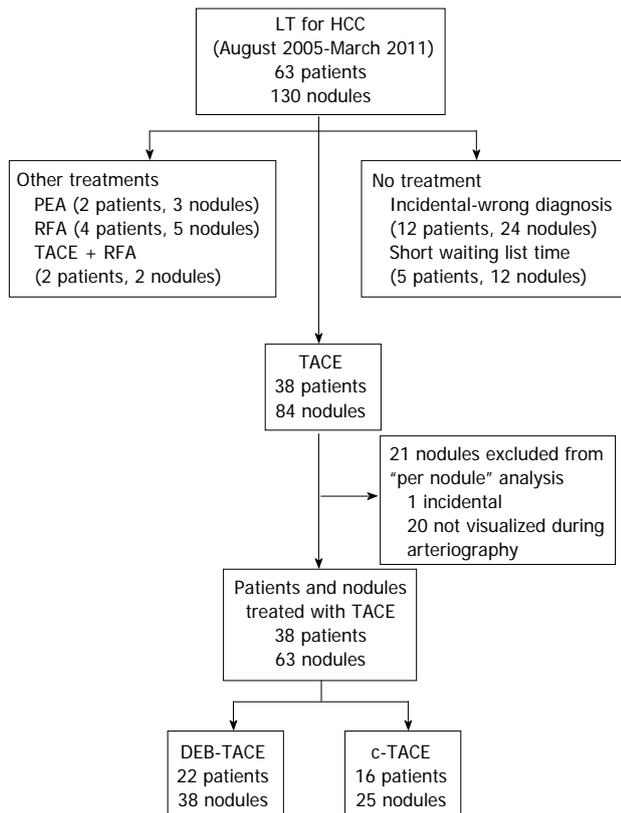
In an intent-to-treat purpose, one of the major limitations of LT in HCC carriers is the time spent on the waiting list; the risk of tumor progression increases with time, resulting in a cumulative probability of dropout from the waiting list of 7.2% for a 6-mo waiting time, which rises to 37.8% and 55.1% for 12 and 18 mo of waiting time, respectively<sup>[6]</sup>.

To attempt to cure to a larger number of HCC carriers, two strategies have been outlined. The first is to downstage those tumors that exceed the Milan Criteria at the time of the first observation, thereby allowing transplantation. The second strategy is to delay the tumor growth using locoregional treatments while the patient is on the waiting list to reduce the dropout rate. The response to locoregional treatment is related to patient prognosis and seems to denote favorable tumor biology<sup>[7-10]</sup>.

Transcatheter arterial chemoembolization (TACE) is the most frequently used treatment of HCC in LT candidates<sup>[11]</sup>. TACE is usually performed by administering a mixture of epirubicin and Lipiodol to concentrate the drug within the tumor. This is followed by a gelatin sponge (conventional TACE, c-TACE) to obtain occlusion of the feeding arteries of the tumor, with the aim of producing infarction and necrosis of the tumor tissue. Recently, a novel doxorubicin-eluting bead (DEB) has been developed to bind, deliver and elute chemotherapeutic drugs in the tumor area during TACE. Pre-clinical and clinical studies have demonstrated that DEB-TACE produces a higher drug concentration within the tumor than c-TACE while maintaining a lower systemic concentration<sup>[12-14]</sup>.

The assessment of the tumor response after TACE remains a critical issue; the Response Evaluation Criteria in Solid Tumors (RECIST), based on the sum of the largest diameter of target lesions on computed tomography (CT) or magnetic resonance (MR) before and during treatment, can be misleading when assessing the treatment-related tumor necrosis, which is not necessarily associated with a reduction in tumor diameter<sup>[15,16]</sup>. In 2001, a panel of experts concluded that an estimation of the reduction of viable tumor (recognized as the non-enhancing areas on a CT scan) should be considered the optimal method to assess local response (modified-RECIST)<sup>[17]</sup>.

However, CT findings often underestimate the residual tumor extent, which can be accurately determined only at histology<sup>[18]</sup>; in this regard, LT represents a unique set-



**Figure 1** Flow chart of the patients included in the study. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; HCC: Hepatocellular carcinoma; LT: Liver transplantation; PEA: Percutaneous ethanol ablation; RFA: Radiofrequency tumor ablation; c-TACE: Conventional TACE.

ting to correctly assess tumor necrosis induced by TACE, as the whole native liver becomes available for histological examination.

Although excellent tumor necrosis rates induced by TACE are reported in LT recipients<sup>[19]</sup>, the impact of TACE on recurrence-free survival remains to be established<sup>[20-22]</sup>.

The aim of this study was to compare DEB-TACE with c-TACE by assessing the histological features of the tumor nodules in native livers removed at transplantation, focusing on the degree of necrosis, as well as to assess the recurrence-free survival of HCC recipients after LT.

## MATERIALS AND METHODS

From August 2005 to March 2011, 63 liver transplants were performed in patients with HCC on cirrhosis in our center. We retrospectively analyzed the histological features of 130 HCC nodules in 63 native livers removed at transplantation (2.06 nodules per patient). Patients who received any other type of treatment, including radiofrequency tumor ablation (RFA) or percutaneous ethanol ablation (PEA), or those who were not treated at all were excluded from the present analysis, as described in Figure 1. Thirty-eight patients who received one or more TACEs as the only neoadjuvant therapy before LT represent the study population.

The policy of our center for TACE is to downstage those tumors that are initially beyond the MC. In the present study, only those patients who were successfully downstaged within the MC and therefore underwent LT are considered; TACE was also performed in those patients who fulfilled the MC and entered the waiting list with an expected waiting time longer than 2 mo. Based on the imaging findings, all the patients in the present study were within the Milan Criteria at the time of transplantation.

As the initial endpoint of our study was to confirm the safety of DEB-TACE and to assess its efficacy in achieving tumor necrosis in comparison with c-TACE, we performed a per nodule analysis of the explanted livers to establish the mean percentage of necrosis of any nodule treated by TACE (conventional or DEB). Twenty-one nodules were not reached by the treatment due to the failure to visualize the tumor’s feeding arteries during arteriography (20 cases) or failure to visualize the tumor itself in the pre-LT imaging (1 case); these 21 nodules were not included in the “per nodule” analysis to assess the mean percentage of necrosis produced by TACE. However, these nodules were taken into account in the “per patient analysis” and in the “survival analysis” to precisely quantify the neoplastic burden of each patient, which could influence the prognosis.

Demographics (age, sex), etiology of cirrhosis, the Child-Pugh and the Model for End Stage Liver Disease (MELD score), radiological and pathological tumor classification according to MC, laboratory tests, imaging studies and pathology reports were recorded for each patient. Factors related to tumor biology, such as serum alpha-fetoprotein, microvascular invasion (MVI) and grading, that play a key role in determining tumor recurrence<sup>[23-26]</sup> were compared between the 2 groups. The waiting time for LT and the interval between the last TACE and LT were also calculated. Computed tomography scans were performed one month after TACE and every 3 mo thereafter; candidates who were initially beyond MC at imaging were reassessed by two interventional radiologist according to the European Association for the Study of the Liver guidelines<sup>[17]</sup> to define the amount of tumor necrosis after TACE. When radiologic findings demonstrated viable tumor beyond MC, chemoembolization was repeated.

In addition to the type of TACE, the impact of the following risk factors on recurrence-free survival was also assessed: adherence to MC at pathology (considering only the viable portion of each nodule), tumor grading (G3-G4), the presence of MVI, the presence of multiple nodules at pathology, a percent necrosis in the cumulative tumor area (CTA) less than or equal to 50%, high levels of  $\alpha$ -fetoprotein (> 70 ng/mL) and the need to repeat TACE before LT.

### Transcatheter arterial chemoembolization

All patients underwent baseline celiac and superior mesenteric arteriography *via* a femoral artery approach. Prior to embolization, liver vascular anatomy was identified

to check the patency of the portal vein and visualize the arterial feeders of the tumor(s). The procedure was defined as “superselective” when the tip of a highly flexible coaxial microcatheter (2.7 Fr; Progreat; Terumo) was successfully placed in the branches supplying the tumor. When nodules were fed by multiple tiny arteries or when multinodular disease was present, TACE was performed with segmental or lobar (only for c-TACE group) catheterization (non-superselective TACE). Conventional TACE was performed by administering a mixture of 50 mg of epirubicin (Pfizer, New York, NY, United States) in an emulsion with lipiodol (Guebert, Aulnay-sous-Bois, France), followed by embolization with gelatin sponge particles (SPONGOSTAN; Johnson and Johnson, Gargrave, United Kingdom). DC beads (Biocompatibles, Farnham, Surrey, United Kingdom) became available at our institution beginning in June 2007; thereafter, patients were randomly assigned to one of the two techniques. The caliber of beads was chosen based on the type of catheterization, the vascularity of the lesion, and the tumor diameter. DEB-TACE was performed using 100- to 300- $\mu\text{m}$  beads for single lesions < 50 mm without arteriovenous shunts, whereas in larger tumors, multiloculated lesions or suspected satellites, one vial of 100- to 300- $\mu\text{m}$  beads and one vial of 300- to 500- $\mu\text{m}$  beads were injected. DC beads were impregnated with 75 mg of doxorubicin in each vial to a maximum of 150 mg of doxorubicin loaded in two vials of DC beads (4 mL total).

### Histopathology

After LT, a dedicated liver pathologist performed the analysis of all the explanted livers, which were serially cut into sections of approximately 0.5 cm in thickness. The presence of cirrhosis was confirmed in all cases. Every lesion suspected to be HCC was completely paraffin-embedded, and multiple histological sections were made. Tumor grade according to the Edmonson and Steiner<sup>[27]</sup> classification and the presence of MVI were also assessed, except when complete necrosis of the tumor was achieved. The necrosis rate of each nodule was expressed as the percentage of necrotic tissue within the whole area of the nodule; necrosis was categorized as complete (100%), appropriate (90% or greater), partial (between 51% and 89%), or inadequate (50% or lower) as described previously<sup>[28,29]</sup>. The sum of the tumor diameters, CTA, and the cumulative necrotic and viable areas (including not-treated nodules) were measured in each patient to calculate the percentage of tumor necrosis within the cumulative tumor area (% necrosis on CTA). Adherence to MC was assessed during the pathological examination using only the viable portion of each nodule. Inflammatory and fibrotic changes in the tissue surrounding the tumor nodule were analyzed and categorized as poor/absent, moderate or enhanced reaction. The localization of microspheres with respect to the 38 HCC nodules treated with DEB-TACE was assessed and defined as intratumoral (exclusively within the tumor capsule), peritumoral (outside the nodule but within 5 mm

of the tumor capsule), intra- and peritumoral or intratumoral (microspheres found in the cirrhotic parenchyma beyond 5 mm from the tumor capsule).

### Statistical analysis

Continuous variables were reported as the mean and standard deviation or as median and range and were compared using Student's *t* test or Mann-Whitney *U* test when appropriate. Categorical variables were reported as numbers and percentages and compared using Fisher's exact test. Recurrence-free survival was calculated from the day of surgery to the first follow-up visit at which tumor recurrence was diagnosed or, in patients without recurrence, to the most recent follow-up visit. Follow-up of those patients who died without evidence of recurrence was censored at the time of death. The impact of each individual variable in determining HCC recurrence-free survival was assessed using the Kaplan-Meier method and compared using the log-rank test. Continuous variables, including pre-LT serum alpha-fetoprotein levels and the percentage of necrosis on CTA, were dichotomized; cutoff values were defined according to receiver operating characteristic curve analysis<sup>[30]</sup>. To identify factors independently related to HCC recurrence, the multivariate Cox proportional-hazard regression analysis was applied, taking in account only the variables that proved significant in the univariate analysis. A two-sided *P* value of less than 0.05 was considered statistically significant in all cases.

## RESULTS

Twenty-two patients underwent DEB-TACE, and 16 underwent c-TACE. Age at LT, gender, etiology and severity of cirrhosis were comparable in the two treatment arms (Table 1).

No major complications were observed after TACE. The post-embolization syndrome (transient fever, abdominal pain, nausea) was the most common complication following chemoembolization in both groups; all side effects were successfully treated with medical therapy. The median time spent on the waiting list was similar in the two groups (3.3 mo in the c-TACE, *vs* 2.9 mo in the DEB-TACE group, respectively, *P* = 0.5844).

DEB-TACE and c-TACE were repeated in 12 (54.5%) and 8 (50%) patients, respectively; the maximum number of treatments per patient was 3. The post-LT mean follow-up time was  $34.9 \pm 19.0$  and  $46.8 \pm 25.6$  mo for the DEB-TACE and c-TACE groups, respectively (*P* = 0.1065). No patients were lost at follow-up.

### Nodule analysis according to the type of TACE

The size and focality of the tumors were comparable in the two groups at explant examination. The mean tumor necrosis was  $55.7\% \pm 41.9\%$  and  $52.2\% \pm 40.9\%$  in DEB- and c-TACE groups, respectively (*P* = 0.7420). A trend towards a higher probability of an appropriate response was observed in the DEB-TACE group (17/38,

**Table 1** Baseline characteristics of the study population *n* (%)

Variable	All treated patients ( <i>n</i> = 38)	Type of TACE		<i>P</i> value
		DEB-TACE ( <i>n</i> = 22)	c-TACE ( <i>n</i> = 16)	
Age at LT (yr)	56.5 ± 6.5	57.2 ± 6.5	55.6 ± 6.5	0.4545
Male gender	34 (89.5)	19 (86.4)	15 (93.7)	0.6245
MELD score	10 (6-27)	9 (6-27)	10 (7-16)	0.4688
Etiology of cirrhosis				0.1834
HCV-related	22 (57.9)	10 (45.5)	12 (75.0)	
HBV-related	11 (28.9)	8 (36.3)	3 (18.7)	
Non-viral	5 (13.2)	4 (18.2)	1 (6.3)	
Waiting list time (mo)	3.1 (0.1-26.7)	2.9 (0.1-24.3)	3.3 (0.6-26.7)	0.5844
Interval between last TACE and LT (mo)	3.6 (0.1-15.9)	2.3 (0.2-13.8)	5.5 (0.9-15.9)	0.0625
Repeated TACE	20 (52.6)	12 (54.5)	8 (50.0)	0.7817
Adherence to MC at imaging before TACE				0.3243
Within MC	21 (55.3)	14 (63.6)	7 (43.7)	
Beyond MC	17 (44.7)	8 (36.4)	9 (56.3)	
BCLC stage before TACE				0.3243
A	21 (55.3)	14 (63.6)	7 (43.7)	
B	17 (44.7)	8 (36.4)	9 (56.3)	
Number of nodules before TACE	2 (1-5)	2 (1-5)	2 (1-5)	0.8708
Nodule number class before TACE				0.2222
1 nodule	16 (42.1)	8 (36.4)	8 (50.0)	
1 < nodules < 4	13 (34.2)	10 (45.4)	3 (18.8)	
Nodules ≥ 4	9 (23.7)	4 (18.2)	5 (31.2)	
<sup>1</sup> Serum α-fetoprotein > 70 ng/mL	8/33 (24.2)	3/18 (16.7)	5/15 (33.3)	0.4811
Post-LT follow-up (mo)	39.9 ± 22.5	34.9 ± 19.0	46.8 ± 25.6	0.1065

Continuous variables are reported as the mean and standard deviation or median and range and compared using Student's *t* test or the Mann-Whitney *U* test as appropriate. Categorical variables are reported as numbers and percentages. <sup>1</sup>Value of serum α-fetoprotein was not available for 5 patients. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; MC: Milan Criteria; LT: Liver transplantation; HCV: Hepatitis C virus; HBV: Hepatitis B virus; MELD: Model for End Stage Liver Disease; c-TACE: Conventional TACE; BCLC: Barcelona Clinic Liver Cancer.

44.7% of nodules) in comparison with the c-TACE group (8/25, 32.0% of nodules), although this difference was not statistically significant ( $P = 0.2834$ ). No difference in necrosis was found comparing the superselective procedures performed with DEB-TACE or c-TACE ( $76.2\% \pm 33.8\%$  vs  $69.1\% \pm 36.5\%$ ,  $P = 0.5803$ ). However, independent of the type of TACE (DEB or conventional), superselective procedures resulted in a higher percentage of necrosis than did non-superselective procedures ( $73.9\% \pm 34.3\%$  vs  $31.3\% \pm 37.0\%$ ,  $P = 0.0018$ ) (Table 2).

Microscopically, tumor necrosis was mixed, colliquative and coagulative in all cases. In the DEB-TACE group only, a "foreign body reaction" with granulomatosis giant cells was observed in the tissue surrounding the tumor in 20 out of 22 patients (90.1%). As described in Figure 2, the nonspecific acute inflammatory infiltrate, containing foamy histiocytes and lymphocytes, was more enhanced at the tumor periphery of DEB-TACE-treated nodules in comparison with the c-TACE-treated ones. In most cases, DEB-TACE-treated nodules were surrounded by thick walls of tissue made of degenerated collagen fibers, inflamed granulation tissue, and hyalinization. The peritumor fibrous tissue of the nodules treated with c-TACE was thinner and apparently less affected by the secondary changes induced by DEB-TACE. These features were not dependent on the time between last treatment and LT; patients with nodules surrounded by an enhanced or mild fibrotic reaction were transplanted  $4.2 \pm 4.0$  mo

after TACE, whereas patients with poor-absent fibrosis were transplanted  $6.0 \pm 4.1$  mo after TACE ( $P = 0.2024$ ).

The distribution of microspheres with respect to the 38 nodules treated by DEB-TACE was intratumoral (11/38, 28.9%), intra- and peritumoral (14/38, 36.8%), peritumoral (10/38, 26.3%) and intratumoral (3/38, 7.9%). Nodules' necrosis differed with respect to the distribution of beads (85.9%, 57.0%, 34.5% and 10.0% for intratumoral, intra- and peritumoral, peritumoral and intratumoral distribution, respectively,  $P = 0.0041$ ).

#### Patient analysis according to the type of TACE

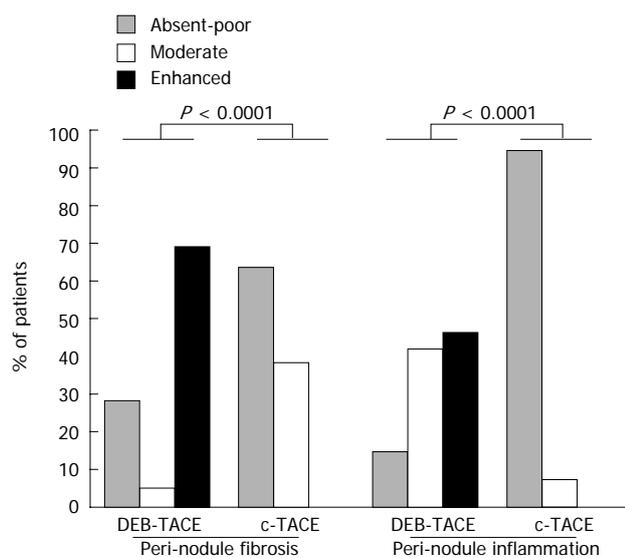
Ten of 17 patients were successfully downstaged within the MC; the effectiveness of DEB-TACE and c-TACE was similar to this regard. Seven out of 17 patients who were beyond MC at the imaging performed before TACE (8 patients in DEB-TACE and 9 in c-TACE group) remained outside the MC at pathology; the failure to accurately stage the tumor during the imaging performed before the LT was related to the misdiagnosis of complete necrosis (Table 3).

The number of nodules that were not reached by TACE (21/84, 25%) was similar in the 2 groups (13/51, 25.4% in DEB-TACE and 8/33, 24.2% in c-TACE group,  $P = 0.8934$ ). The mean diameter of the missed nodules was  $1.1 \pm 0.5$  cm, and 10 out of these 21 nodules (47.6%) were equal or inferior to 1 centimeter in size. The number of HCC nodules, the sum of the tumor diameters and the CTA (also including untreated nodules)

**Table 2** Analysis of the treated nodules according to the type of transcatheter arterial chemoembolization (per nodule analysis) *n* (%)

Variable	All treated nodules 63 in 38 patients	Type of TACE		P value
		DEB-TACE 38 in 22 patients	c-TACE 25 in 16 patients	
Degree of necrosis	54.3% ± 41.2%	55.7% ± 41.9%	52.2% ± 40.9%	0.7420
Complete necrosis (100%)	21 (33.3)	14 (36.8)	7 (28.0)	0.5877
Histological response				0.2834
Appropriate (necrosis ≥ 90%)	25 (39.7)	17 (44.7)	8 (32.0)	
Partial (50% < necrosis < 90%)	9 (14.3)	3 (7.9)	6 (24.0)	
Inadequate (necrosis ≤ 50%)	29 (46.0)	18 (47.4)	11 (44.0)	
Diameters of nodules (cm)	2 (0.7-10)	1.8 (0.7-4.5)	2.2 (1-10)	0.1752
Number of nodules				0.2492
Single	17 (27.0)	8 (21.1)	9 (36.0)	
Degree of necrosis	63.1% ± 37.8%	69.7% ± 34.8%	57.2% ± 41.5%	0.5144
Multiple	46 (73.0)	30 (78.9)	16 (64.0)	
Degree of necrosis	51.1% ± 42.3%	52.0% ± 43.4%	49.4% ± 41.7%	0.8454
Modality of TACE				0.3015
Superselective	34 (54.0)	23 (60.5)	11 (44.0)	
Degree of necrosis	73.9% ± 34.3%	76.2% ± 33.8%	69.1% ± 36.5%	0.5803
Non-superselective	29 (46.0)	15 (39.5%)	14 (56.0)	
Degree of necrosis	31.3% ± 37.0%	24.3% ± 33.3%	38.9% ± 40.5%	0.2970

Analysis performed considering nodules reached by transarterial treatment (targeted lesions). Continuous variables are reported as median and range or mean and standard deviation and compared using the Student's *t* test or Mann-Whitney *U* test as appropriate. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; c-TACE: Conventional TACE.



**Figure 2** Inflammatory and fibrotic changes in the tissue surrounding the tumor nodules. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead.

were similar in the two groups. The mean percentage of necrosis on CTA was 58.8% and 50.2% in the DEB- and c-TACE group, respectively ( $P = 0.4856$ ); no difference in terms of response to treatment in various subcategories was observed. Risk factors for recurrence, such as a pre-transplant serum  $\alpha$ -fetoprotein greater than 70 ng/mL (Table 1), tumor grading and microvascular invasion were similarly distributed in the two treatment arms.

### Recurrence and survival after LT

Overall 3-year survival after LT was 73.9% and 58.7% in the DEB-TACE and c-TACE groups, respectively ( $P$

$= 0.7511$ ). Seven (18.4%) patients experienced tumor recurrence after LT; the main site of recurrence was the liver (5 patients), the spinal cord and the liver concurrently (1 patient) and the adrenal gland (1 patient). The mean time to recurrence was  $17.0 \pm 5.5$  mo; three out of seven patients were alive with recurrence at the time of publication.

The three-year recurrence-free survival was significantly higher in patients who were treated preoperatively with DEB-TACE than with c-TACE (87.4% *vs* 61.5%,  $P = 0.0493$ , Figure 3A).

Other factors affecting recurrence-free survival included Milan Criteria unfulfilled at pathology, percentage of necrosis on CTA lower than 50% and pre-transplant serum  $\alpha$ -fetoprotein levels greater than 70 ng/mL (Figure 3B-D). On multivariate analysis, a lack of treatment with DEB-TACE, serum  $\alpha$ -fetoprotein levels exceeding 70 ng/mL and Milan Criteria unfulfilled at pathology were independent predictors of tumor recurrence (Table 4).

## DISCUSSION

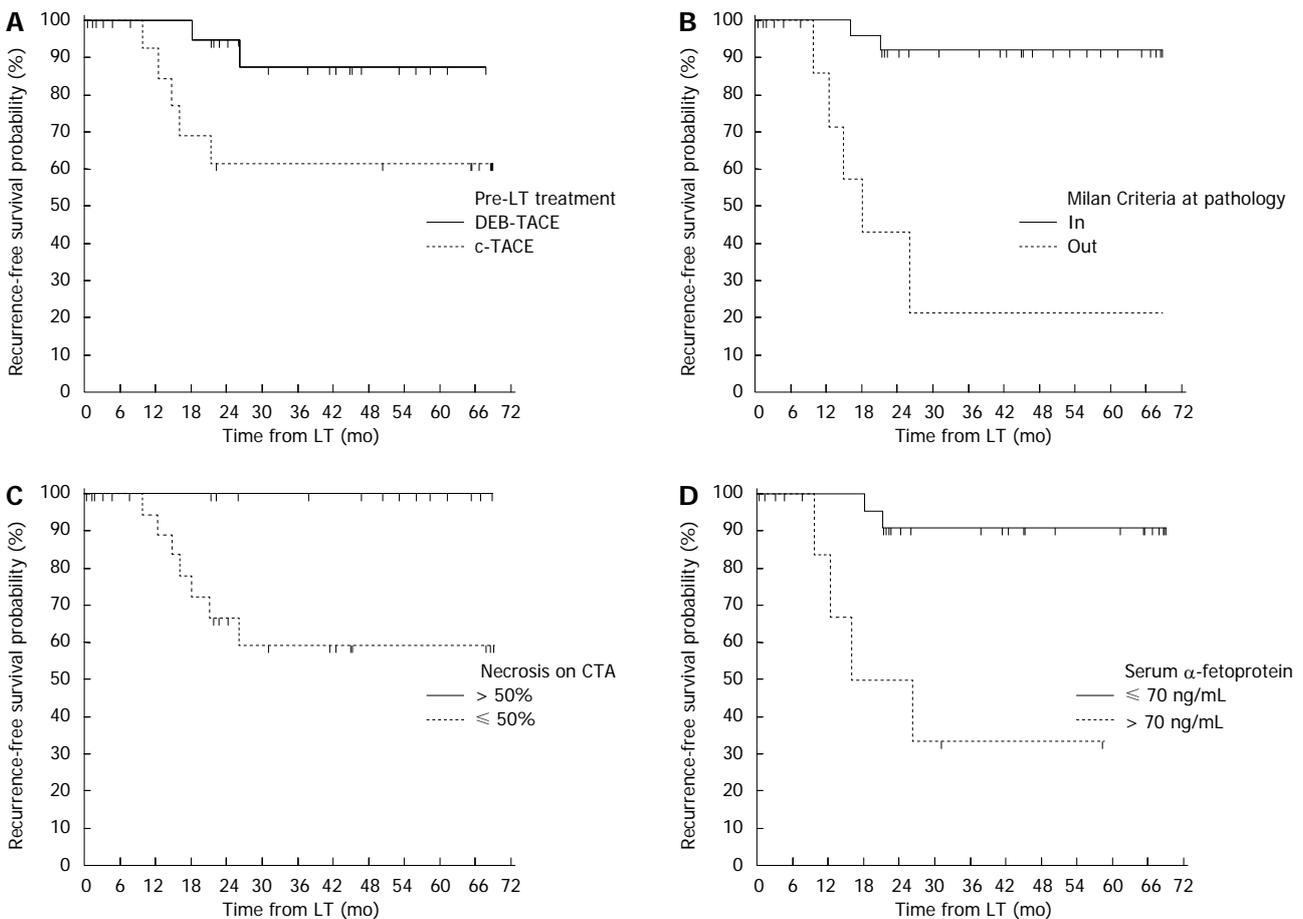
Among patients with HCC awaiting LT, TACE is the most commonly used neo-adjuvant therapy<sup>[11]</sup>. Although TACE can successfully downstage 24% to 63% of HCCs, pre-LT treatment is not clearly associated with any survival benefit<sup>[22,31-33]</sup>; in a large multicenter study, the 5-year recurrence-free survival was 67% in patients treated with TACE prior to LT and 64% in those not treated<sup>[20]</sup>.

Unlike conventional TACE, which is the most commonly used technique, DEB-TACE is based on calibrated microspheres made of non-degradable polymers that produce permanent vascular embolization and

**Table 3 Patient analysis according to the type of transcatheter arterial chemoembolization (per patient analysis) *n* (%)**

Variable	All treated patients ( <i>n</i> = 38)	Type of TACE		P value
		DEB-TACE ( <i>n</i> = 22)	c-TACE ( <i>n</i> = 16)	
Number of nodules per patients	2.2 ± 1.3	2.2 ± 1.2	2.1 ± 1.4	0.7019
Untreated nodules	21/84 (25.0)	13/51 (25.4)	8/33 (24.2)	0.8934
Nodule number class at pathology				0.1473
1 nodule	17 (44.7)	8 (36.4)	9 (56.3)	
1 < nodules < 4	14 (36.8)	11 (50.0)	3 (18.7)	
≥ 4 nodules	7 (18.4)	3 (13.6)	4 (25.0)	
Sum of tumor diameters (cm)	4.2 ± 2.8	4.1 ± 2.4	4.5 ± 3.3	0.5813
CTA (cm <sup>2</sup> )	5.5 (0.8-78.5)	4.6 (1.5-19.1)	7.2 (0.8-78.5)	0.8592
Necrosis on CTA	55.2% ± 37.0%	58.8% ± 36.6%	50.2% ± 38.1%	0.4856
Adherence to MC at pathology				0.4250
Within MC	31 (81.6)	19 (86.4)	12 (75.0)	
Beyond MC	7 (18.4)	3 (13.6)	4 (25.0)	
Histological response				0.2896
Appropriate (necrosis on CTA ≥ 90%)	11 (28.9)	8 (36.4)	3 (18.7)	
Partial (50% < necrosis on CTA < 90%)	8 (21.1)	3 (13.6)	5 (31.3)	
Inadequate (necrosis on CTA ≤ 50%)	19 (50.0)	11 (50.0)	8 (50.0)	
Risk factors for recurrence				
Microvascular invasion	7 (18.4)	5 (22.7)	2 (12.5)	0.4271
<sup>1</sup> Grading > 2	7 (18.4)	2/18 (11.1)	5/14 (35.7)	0.1948

Analysis includes the 21 nodules not reached by transarterial treatment (non-targeted lesions). Continuous variables are reported as medians and ranges or means and standard deviations. <sup>1</sup>Assessment of tumor grading was not available for 6 patients who had complete necrosis at explant examination. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; CTA: Cumulative tumor area; MC: Milan Criteria; c-TACE: Conventional TACE.



**Figure 3 Recurrence-free survival probabilities according to the following.** A: Pre-transplant treatment type (log-rank *P* = 0.0493); B: Adherence to Milan Criteria at pathology (log-rank *P* < 0.0001); C: Percentage of necrosis in the cumulative tumor area (log-rank *P* = 0.0098); D: Pre-transplant serum  $\alpha$ -fetoprotein level (log-rank *P* = 0.0008). TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; LT: Liver transplantation; CTA: Cumulative tumor area; c-TACE: Conventional TACE.

**Table 4** Univariate and multivariate analysis of risk factors related to tumor recurrence

Risk factors	Univariate analysis			Multivariate analysis	
	3-yr recurrence-free survival rate	HR (95%CI)	Log rank P value	Exp(B) (95%CI)	P value
Tumor grading G3-G4 ( <i>vs</i> G1-G2)	66.7% <i>vs</i> 74.2%	1.76 (0.289-13.149)	0.4934		
Presence of microvascular invasion ( <i>vs</i> absence)	60.0% <i>vs</i> 80.7%	2.74 (0.451-39.277)	0.2071		
Multiple nodules at pathology ( <i>vs</i> single)	69.1% <i>vs</i> 85.7%	2.15 (0.460-9.070)	0.3478		
Repeated TACE ( <i>vs</i> single)	74.1% <i>vs</i> 80.0%	1.08 (0.244-4.821)	0.9148		
Necrosis on CTA $\leq$ 50% ( <i>vs</i> > 50%)	59.3% <i>vs</i> 100.0%	NA <sup>1</sup>	0.0098	NA <sup>1</sup>	NA <sup>1</sup>
MC unfulfilled at pathology ( <i>vs</i> fulfilled)	21.4% <i>vs</i> 92.0%	13.84 (10.121-636.416)	< 0.0001	11.6 (1.932-69.646)	0.0077
Absence of DEB-TACE ( <i>vs</i> presence)	61.5% <i>vs</i> 87.4%	4.47 (1.005-22.188)	0.0493	15.45 (1.457-163.766)	0.0237
$\alpha$ -fetoprotein > 70 ng/mL ( <i>vs</i> $\leq$ 70 ng/mL)	33.3% <i>vs</i> 90.9%	10.31 (4.749-370.242)	0.0008	15.31 (1.766-132.614)	0.0137

<sup>1</sup>Heart rate calculation and the inclusion of the covariate in the multivariate analysis was not applicable due to the lack of hepatocellular carcinoma recurrence events in necrosis on the cumulative tumor area (CTA) > 50% group. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; MC: Milan Criteria; NA: Not available.

increase intra-tumor drug delivery. There are 3 substantial pharmacokinetic advantages associated with DEB-TACE: a continuous elution of the drug for prolonged time, a higher concentration locally into the tumor and a lower systemic exposure to the drug in comparison with c-TACE<sup>[12]</sup>. In a preclinical study, Hong *et al* demonstrated that the peak of doxorubicin within the tumor is registered after three days, and drug levels remain high up to fourteen days after treatment<sup>[14]</sup>.

Several clinical studies have compared DEB and conventional TACE in non-transplant settings. A recent randomized study including 212 patients failed to demonstrate a significant difference in the overall radiological response, although a better safety profile and a trend toward a better response rate was observed for DEB-TACE<sup>[13]</sup>. Malagari *et al*<sup>[34]</sup> demonstrated that DEB-TACE was able to stabilize disease in a higher percentage of patients when compared with bland embolization (embolic agents without drug), but the survival rate at 12 mo did not differ in the two groups. In another prospective study, complete and partial response rates, tumor recurrence and overall survival were similar with DEB-TACE and conventional TACE<sup>[35]</sup>. Although a retrospective study recently suggested a higher 2-year survival rate in DEB-TACE patients<sup>[36]</sup>, the superiority of this technique remains to be further investigated.

Liver transplant candidates exhibit completely different characteristics than those patients considered in the above-mentioned studies. First, HCC in LT candidates is not advanced. Furthermore, the response to TACE in terms of tumor necrosis has clinical relevance only in those patients who require downstaging, whereas in the others, the goal is to halt tumor progression. Last, recurrence-free survival, measured following LT, is a realistic endpoint, as in the non-transplant setting, TACE is not intended to be curative<sup>[37]</sup>.

Few reports are available about the results of DEB-TACE in LT candidates. A small study from Milan reported a higher complete histological necrosis rate (77%) in patients treated with DEB-TACE. However, only 8 patients had been treated with DEB-TACE in that study,

while the 8 patients of the control group received bland embolization (non-loaded microspheres), with very low complete necrosis rates (27.2%)<sup>[38]</sup>. Unlike from the present study, Farris *et al*<sup>[39]</sup> recently reported a significantly higher necrosis rate after c-TACE in comparison with DEB-TACE (66.4% *vs* 46.1%). However, no mention of the HCC recurrence-free survival of the patients was made in these studies.

In our series, histopathological examination of the native livers did not indicate a significant difference between DEB-TACE and conventional TACE with regard to the effectiveness of the two different procedures in inducing histological necrosis and achieving tumor downstaging. However, a peculiar histological pattern was associated with DEB-TACE; DEB-TACE was characterized by an intense inflammatory and fibrotic reaction in the area surrounding the tumor tissue that was not observed in those patients treated with conventional TACE. Remarkably, a lower tumor recurrence rate after LT was associated with DEB-TACE. Furthermore, DEB-TACE was identified as an independent predictor of recurrence-free survival in the multivariate analysis. The others independent prognostic determinants found in the present study, serum alpha-fetoprotein levels and adherence to MC at histopathological examination, have been previously identified by others to be strictly linked with HCC recurrence after LT<sup>[3]</sup>.

Pretransplant ablative treatments have the potential to decrease the release and growth of HCC metastases. As the release of tumor cells can be intermittent, the continuous elution of doxorubicin and the distribution of loaded beads in the vessels around the nodule might maintain a prolonged antineoplastic effect and explain the lower recurrence rate observed in the DEB-TACE group. The biological significance of the intense tissue reaction that surrounds the tumor treated with DEB-TACE must to be further investigated. However, one might speculate that the tissue reaction could play a role in limiting tumor spread.

The difference in the chemotherapeutic agent employed in the two different TACE techniques (epirubicin

in c-TACE and doxorubicin in DEB-TACE) is unlikely to have influenced the results; in a large randomized controlled trial that compared c-TACE made using a lipiodol emulsion containing epirubicin or doxorubicin, no difference in the incidence of adverse reactions, changes in alpha-fetoprotein, extent of tumor reduction or the survival rates between the two drugs was reported<sup>[40]</sup>.

Although further confirmation of our findings with randomized controlled trials is warranted, our report seems to indicate that the use of DEB-TACE in LT recipients with HCC can increase recurrence-free survival after liver transplantation.

## COMMENTS

### Background

Transcatheter arterial chemoembolization (TACE) is the most common locoregional treatment in cirrhotic patients with hepatocellular carcinoma (HCC) awaiting liver transplantation (LT), and the main objective of TACE is to prevent tumor progression in HCC patients who have already met the Criteria for transplantation or to downstage tumors initially outside Milan Criteria to allow LT. In addition to conventional TACE (c-TACE), based on mixtures of anticancer drugs, lipiodol and a gelatin sponge, a procedure with calibrated doxorubicin-loaded microspheres has been recently developed (DEB-TACE). In pre-LT setting, only a few reports have compared the impact of different TACE regimens on tumor histology and recurrence-free survival after transplantation.

### Research frontiers

Pre-clinical and clinical studies have demonstrated that DEB-TACE produces a higher drug concentration within the tumor than does c-TACE in presence of a lower systemic concentration, but its superiority in inducing tumor necrosis and increasing recurrence-free survival remains to be further investigated. As the whole native liver becomes available for histological examination, LT represents a unique setting to correctly assess necrosis and histological changes in tumor nodules of patients treated by TACE. This approach can be useful in developing new strategies to decrease the release of HCC metastases in patients awaiting LT.

### Innovations and breakthroughs

A lower tumor recurrence rate after LT was observed in patients who were treated preoperatively with DEB-TACE. Although no significant differences were observed in terms of tumor necrosis between DEB and c-TACE, a peculiar histological pattern was associated with DEB-TACE, characterized by an intense inflammatory and fibrotic reaction in the area surrounding the tumor tissue. This finding, in addition to the prolonged antineoplastic effect of loaded beads in the vessels around the nodule, could limit tumor spread during time on the waiting list and could explain the lower postoperative recurrence rate observed in the DEB-TACE group.

### Applications

According to the results of this study, DEB-TACE is an effective locoregional tool for the management of HCC patients awaiting liver transplantation and can increase recurrence-free survival after LT.

### Terminology

TACE indicates transcatheter arterial chemoembolization. DEB-TACE indicates TACE with calibrated, doxorubicin-loaded microspheres used to bind, deliver and elute chemotherapeutic drugs in the tumor area. c-TACE indicates the conventional TACE procedure, performed by administering a mixture of epirubicin in an emulsion with lipiodol followed by a gelatin sponge to obtain occlusion of the feeding arteries of the tumor. HCC indicates hepatocellular carcinoma. LT indicates liver transplantation.

### Peer review

The authors present an interesting retrospective single-center study that clearly addresses pre-LT treatment of HCC. The authors highlight an important locoregional therapeutic tool, DEB-TACE, which has become increasingly utilized and can improve the outcomes of LT for HCC. The explanted livers underwent very close pathological scrutiny to judge the effects of the 2 different therapies; data analysis is well done. The paper is well written, and the manuscript improves significantly on the knowledge of the role of DEB-TACE in the management of HCC.

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## Protective effect of naringenin on acetic acid-induced ulcerative colitis in rats

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### Abstract

**AIM:** To evaluate the ameliorative effect of naringenin (NG) during ulcerative colitis (UC) in rats.

**METHODS:** Rats were treated with three different doses (25, 50 and 100 mg/kg per day) of NG and a single dose of mesalazine (MES, 300 mg/kg per day) for seven days prior to ulcerative colitis induction by 4% acetic acid (AA). Twenty four hours after AA rectal administration, animals were scarified and the colonic tissues were dissected. Colonic mucus content was estimated using Alcian blue dye binding technique. In colon tissues, levels of total glutathione sulphadryls (T-

GSH), non-protein sulphadryls (NP-SH) and thiobarbituric acid reactive substances (TBARS) were evaluated. The activities of the antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD) were measured. Concentrations of nucleic acids (DNA and RNA) and total protein were also estimated in colon tissues. Colonic levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO) were estimated. In cross section of colitis tissue the histopathological changes were observed.

**RESULTS:** Colonic mucus content was decreased in AA compared to controls ( $587.09 \pm 65.59$  mg/kg *vs*  $941.78 \pm 68.41$  mg/kg,  $P < 0.001$ ). AA administration markedly reduced T-GSH ( $5.25 \pm 0.37$  nmol/L *vs*  $3.04 \pm 0.24$  nmol/L,  $P < 0.01$ ), NP-SH ( $3.16 \pm 0.04$  nmol/L *vs*  $2.16 \pm 0.30$  nmol/L,  $P < 0.01$ ), CAT ( $6.77 \pm 0.40$  U/mg *vs*  $3.04 \pm 0.2$  U/mg,  $P < 0.01$ ) and SOD ( $3.10 \pm 0.11$  U/mg *vs*  $1.77 \pm 0.18$  U/mg,  $P < 0.01$ ) while TBARS, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, PGE<sub>2</sub> and NO levels ( $15.09 \pm 3.84$  nmol/L *vs*  $59.90 \pm 16.34$  nmol/L,  $P < 0.01$ ;  $113.56 \pm 1.91$  pg/mg *vs*  $134.24 \pm 4.77$  pg/mg,  $P < 0.01$ ;  $209.20 \pm 36.38$  pg/mg *vs*  $422.19 \pm 31.47$  pg/mg,  $P < 0.01$ ;  $250.83 \pm 25.09$  pg/mg *vs*  $638.58 \pm 115.9$  pg/mg,  $P < 0.01$ ;  $248.19 \pm 36.98$  pg/mg *vs*  $541.74 \pm 58.34$  pg/mg,  $P < 0.01$  and  $81.26 \pm 2.98$  mmol/g *vs*  $101.90 \pm 10.73$  mmol/g,  $P < 0.001$ ) were increased in colon of rats with UC compared controls respectively. Naringenin supplementation, significantly and dose dependently increased the colonic mucus content. The elevated TBARS levels were significantly decreased ( $39.35 \pm 5.86$  nmol/L,  $P < 0.05$ ;  $26.74 \pm 3.17$  nmol/L,  $P < 0.01$  nmol/L and  $17.74 \pm 2.69$  nmol/L,  $P < 0.01$ ) compared to AA ( $59.90 \pm 16.34$  nmol/L) group while the decreased levels of T-GSH and NP-SH and activities of CAT and SOD found increased by NG treatments in dose dependent manner. The decreased values of nucleic acids and total protein in AA group were also significantly ( $P < 0.01$ ) increased in all three NG supplemented groups

respectively. NG pretreatment inhibited the TNF- $\alpha$  levels ( $123.76 \pm 3.76$  pg/mg,  $122.62 \pm 3.41$  pg/mg and  $121.51 \pm 2.61$  pg/mg *vs*  $134.24 \pm 4.78$  pg/mg,  $P < 0.05$ ) compared to AA group, respectively. Interleukins, IL-1 $\beta$  and IL-6 levels were also decreased in NG50 + AA ( $314.37 \pm 16.31$  pg/mg and  $292.58 \pm 23.68$  pg/mg,  $P < 0.05$ ) and NG100 + AA ( $416.72 \pm 49.62$  pg/mg and  $407.96 \pm 43.87$  pg/mg,  $P < 0.05$ ) when compared to AA ( $352.46 \pm 8.58$  pg/mg and  $638.58 \pm 115.98$  pg/mg) group. Similar decrease ( $P < 0.05$ ) was seen in PGE<sub>2</sub> and NO values when compared to AA group. The group pretreated with MES, as a reference drug, showed significant ( $P < 0.01$ ) protection against the changes induced in colon tissue by AA administration respectively.

**CONCLUSION:** In present study, NG produced antioxidant and anti-inflammatory effects demonstrating protective effect in inflammatory bowel disease.

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**Key words:** Naringenin; Ulcerative colitis; Inflammatory bowel disease; Oxidative stress

**Core tip:** Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), results in substantial morbidity and is difficult to treat. New strategies for adjunct therapies are needed. Systemic corticosteroids are highly effective at inducing clinical remission in cases of acute exacerbation of CD and UC; however, their use is limited by their frequent and sometimes severe side effects. Results of present study revealed that, naringenin has protective effects against acetic acid-induced UC by inhibiting inflammatory and oxidative bio-markers. Thus, it may pose promising outcomes for future clinical usage as a natural non-toxic effective supplement in IBD.

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a common chronic inflammatory disease of the gastrointestinal tract. Several etiological factors, such as genetic, immunological, and environmental have been linked with the pathophysiology of the disease<sup>[1]</sup>. There are two main subtypes of IBD; Crohn's disease (CD) and ulcerative colitis (UC) having a combined prevalence of 150-250/100000 population<sup>[2,3]</sup>. Moreover, the prevalence of hospitalization due to CD and UC is estimated to be 50.1 and 50.6 per 100000 population, respectively<sup>[4]</sup>. UC involves only the colon and rectum. Al-

though the etiology of UC is not completely understood, it has been commonly associated with reduced antioxidant capacity as well as increased free radical production such as reactive oxygen species (ROS)<sup>[5]</sup>. Over production of ROS leads to lipid peroxidation (LPO), which can inhibit cellular antioxidant capability finally resulting in prominent colonic inflammation<sup>[6]</sup>. Clinically, colitis patients were found to overproduce ROS and nitrogen species leading to LPO of membranes and attack on tissue proteins and DNA<sup>[7,8]</sup>. Endogenous antioxidant defenses against ROS production even in low concentrations influence on two main types: (1) enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT); and (2) non-enzymatic such as glutathione (GSH) and ascorbic acid (vitamin C). It is suggested that inflammatory response amplification can induce inflammatory cells chemotaxis resulting in release of ROS and inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6 and IL-1 $\beta$ , which triggers the pathological responses and symptoms during IBD<sup>[9]</sup>. Elevated levels of pro-inflammatory cytokines in both the IBD forms reported to have a vital role of such mediators, which also play in determining the severity of the disease<sup>[10]</sup>. Medications that have ability to inhibit the production of these inflammatory mediators are shown to be clinically effective, which indicate their contribution to IBD and other chronic inflammatory conditions aggravation<sup>[11]</sup>.

Some natural products, such as flavonoids, are getting more attention as novel agents for therapeutically usage. Flavonoids are one of the most abundant natural antioxidants present in plants and the human diets. Naringenin (4,5,7-trihydroxy flavanone) a flavonoid, widely distributed in citrus fruits, tomatoes, cherries, and cocoa<sup>[12]</sup>. Several pharmacological studies revealed its effects including antidiabetic<sup>[13]</sup>, antiatherogenic<sup>[14]</sup>, antidepressant<sup>[15]</sup>, immunomodulatory<sup>[14]</sup>, antitumor<sup>[16]</sup>, DNA protective<sup>[17]</sup>, hypolipidemic<sup>[18]</sup> and peroxisome proliferator-activated receptors activator<sup>[19]</sup>. It has also been shown to have prominent antioxidant<sup>[20]</sup> and anti-inflammatory<sup>[21]</sup> potentials. Inês Amaro *et al.*<sup>[22]</sup> reported that, naringenin (NG) has reducing effect on intestinal edema-induced by dextran sodium sulphate in mice.

In several studies, pathogenesis of UC disease has demonstrated that excessive inflammation and oxidative stress play a significant role<sup>[23,24]</sup>. Amelioration of LPO as well as free radicals scavenging would provide a useful, protective and therapeutic treatment for UC. With respect to the high antioxidant capacity and anti-inflammatory activity, NG would be expected to reduce injury and/or improve tissue healing following injury from ulcerative colitis. In the present study we had evaluated the protective effect of NG during experimental ulcerative colitis and the possible mechanism of action.

## MATERIALS AND METHODS

### *Animals and ethical approval*

Eight weeks old male Wister albino rats weighting 250-280 g were received from Experimental Animal

Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were housed under controlled environmental conditions (25 °C and a 12 h light/dark cycle). Animals had free access to Purina rat chow (Manufactured by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and tap water. All experimental procedures and protocols in this study including euthanasia were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) as well as the Ethical Guidelines of the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

### Induction of ulcerative colitis

Experimental ulceration in colon tissue was done according to the method described by Mousavizadeh *et al.*<sup>[25]</sup> with slight modification. In brief, under light ether anesthesia rats were administered 2 mL of 4% acetic acid solution (v/v; Merck, Darmstadt, Germany) by transrectally using a (2.7 mm) soft pediatric catheter. After AA administration, rats were holed horizontally for 2 min to prevent AA leakage. Control animals underwent the same procedure using equal volume of normal saline instead of AA solution.

### Experimental design

Forty two rats were divided into seven groups (six animals in each) as follows: (1) Control (Cont); (2) NG 100 mg/kg per day (NG100); (3) AA treated rats (AA); (4) NG 25 mg/kg per day + acetic acid (NG25 + AA); (5) NG 50 mg/kg per day + AA (NG50 + AA); (6) NG 100 mg/kg per day + AA (NG100 + AA); and (7) MES 300 mg/kg per day + AA (MES + AA). Naringenin (Sigma Aldrich, United States) and MES treatments were continued for 7 consecutive days by gavage<sup>[26]</sup>. At end of the treatment, ulcerative colitis was induced in all AA groups. Twenty four hours after the colitis induction, animals were sacrificed under deep anesthesia<sup>[27]</sup>. The colon (5-6 cm) specimens were dissected, washed with saline solution, imaged, weighted and small cross section was fixed in 10% formaldehyde solution for histopathological evaluation. The remaining tissues were stored at -75 °C (Ultra-low freezer, Environmental Equipment, Cincinnati, Ohio, United States) till analysis.

### Evaluation of the adherent colonic mucus

The modified procedure of Popov *et al.*<sup>[28]</sup> was used to determine adherent colonic mucus concentration. Briefly, a small portion of colon tissue was excised, weighted then transferred immediately to 1% Alcian blue solution (in 0.16 mol/L sucrose solution buffered with sodium acetate, pH 5) for 24 h. The excess dye was removed by rinsing with sucrose solution. The dye complexed with the gastric wall mucus was extracted using 10 mL of 0.5 mol/L MgCl<sub>2</sub> solution. A 4 mL aliquot of blue extract was then shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 rpm and the absorbance of the aqueous layer was recorded at 580

nm by using spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The quantity of Alcian blue extracted (µg) per grams of wet colonic tissue was then calculated.

### Histopathological investigations

Colon sections were fixed 10% neutral buffered formalin then put for 24 h in decal. Samples were then cut into several sections and embedded into paraffin wax blocks. Tissues were stained with haematoxylin and eosin and were mounted and observed microscopically for histopathological changes by a pathologist in blinded fashion.

### Estimations of total glutathione sulphadryls and non-protein sulphadryls concentrations in colon

In colon tissues, total glutathione sulphadryls (T-GSH) and non-protein sulphadryls (NP-SH) levels were estimated according to the method described by Sedlak *et al.*<sup>[29]</sup>. Tissues were homogenated in ice-cold 0.02 mol/L ethylenediamine tetra-acetic acid. An aliquots of 0.5 mL of tissue homogenate was mixed with 0.2 mol/L Tris buffer, pH 8.2 and 0.1 mL of 0.01 mol/L Ellman's reagent, [5,5'-dithiobis-(2-nitr-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 rpm at room temperature for 15 min the absorbance of the clear supernatant was measured using spectrophotometer (LKB-Pharmacia, Mark II, Ireland) at 412 nm. For NP-SH estimation, homogenate was diluted with distilled H<sub>2</sub>O and mixed with 1 mL of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at approximately 3000 g. Two milliliter of supernatant was then added to 4 mL of 0.4 mol/L Tris buffer (pH 8.9) then 0.1 mL DTNB added. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank.

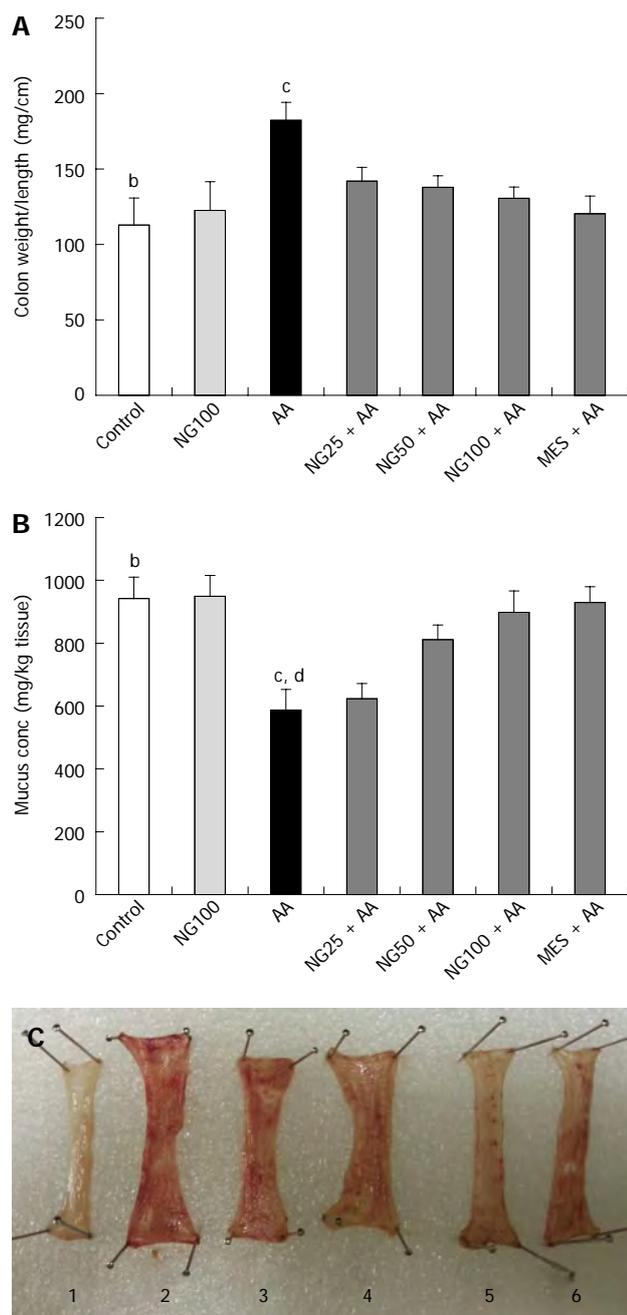
### Estimation of thiobarbituric acid reactive substances levels in colon

A thiobarbituric acid reactive substances (TBARS) assay kit (ZeptoMetrix, United States) was used to measure the LPO products, malondialdehyde (MDA) equivalents. Briefly, one hundred microliters of colon homogenate was added to 2.5 mL reaction buffer (provided by the kit) and heated at 95 °C for 60 min. After the mixture cooling, supernatant absorbance was measured at 532 nm using a spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The LPO products are expressed in terms of nmoles MDA/mg protein.

### Estimation of CAT and SOD activities in colon

Catalase activity in colon tissues was estimated by the method described by Aebi<sup>[30]</sup>. In brief, aliquot of 0.5 mL post-mitochondrial supernatant was mixed with 2.5 mL of 50 mmol/L phosphate buffer (pH 7.0) and 20 mmol/L H<sub>2</sub>O<sub>2</sub>. CAT activity was estimated spectrophotometrically following the decrease in absorbance at 240 nm and expressed in terms of units/mg protein as compared to a standard curve.

The SOD activity in colon tissue was measured by using the method described by Kono<sup>[31]</sup>. The principle of this method was that superoxide anions generated by the



**Figure 1** Effect of naringenin on colon weight/length (A), mucus concentration (B) and induction of ulceration and its protection by treatments in colonic tissue of rats with acetic acid-induced ulcerative colitis (C) ( $n = 6$ ). Values in (A) and (B) are expressed as mean  $\pm$  SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. <sup>b</sup> $P < 0.01$  control vs acetic acid (AA); <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  AA vs naringenin (NG) 25 + AA, NG50 + AA, NG100 + AA or mesalazine (MES) + AA groups. Groups in (C) are arranged as follows: control (1), AA (2), NG25 + AA (3), NG50 + AA (4), NG100 + AA (5) and MES + AA (6).

oxidation of hydroxylamine hydrochloride can mediate the reduction of nitrobluetetrazolium to blue formazon. The color was then measured at 560 nm under aerobic conditions. Addition of superoxide dismutase inhibited nitrobluetetrazolium reduction and the extent of this inhibition was taken as a measure of enzymatic activity. The SOD activity was expressed as units/mg protein.

### Determination of nucleic acids and total protein levels in colon

The method described by Bregman<sup>[32]</sup> was used to determine the levels of nucleic acids (DNA and RNA) in colon. In brief, colon tissues were homogenized in 4 mL ice-cold distilled water and 2 mL homogenate was suspended in 5 mL of 10% ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was extracted twice with 95% ethanol. Finally, the nucleic acids were extracted in 5% TCA. DNA was determined by treating the nucleic acid extract with diphenylamine reagent and measuring the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol reagent and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present. Total protein in colon was estimated by Lowry *et al.*<sup>[33]</sup> method using Bovine plasma albumin as a standard.

### Determination of inflammatory cytokines, PGE<sub>2</sub> and NO levels in colon

In colon, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and PGE<sub>2</sub> levels were assessed and quantified according to the method of Mousavizadeh *et al.*<sup>[25]</sup> using enzyme-linked immunosorbent assay ELISA (R and D systems, United States). The results were expressed as pg/mg tissue. Levels of colonic nitric oxide were assayed by Griess reaction method using commercial kit (R and D systems, United States).

### Statistical analysis

Data were expressed as mean  $\pm$  SE. Statistical analysis was carried out using one-way ANOVA followed by Newman-Keuls *post hoc* test.  $P$  value of  $\leq 0.05$  was considered statistically significant. All statistics tests were conducted using Graph Pad Prism (version 5) software.

## RESULTS

Acetic acid significantly ( $P < 0.01$ ) increased the colonic weight as compared to control group. Pretreatment with NG following three doses and MES for 7 d, showed significant ( $P < 0.05$ ) inhibition in weight increase while compared to AA group (Figure 1A). Mucus concentration was significantly ( $P < 0.01$ ) reduced in AA administered group when compared to control animals. Pretreatment with higher doses (50 and 100 mg/kg) of NG and MES significantly elevated the reduced colonic mucus concentration ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.05$ , respectively) as compared to AA group (Figure 1B). The colon images were clearly showed the induction of ulceration and its protection by the treatments (Figure 1C).

Histopathological changes with their intensity are presented in Figure 2. Slide from control group, showing benign mucosal epithelium of tall columnar epithelial cells with goblet cells (Table 1 and Figure 2A). In the AA group, the slide revealed diffused active colitis with widely eroded mucosa with ulcerations and necrosis associated with edema, goblet cell hyperplasia, lymphoid follicular

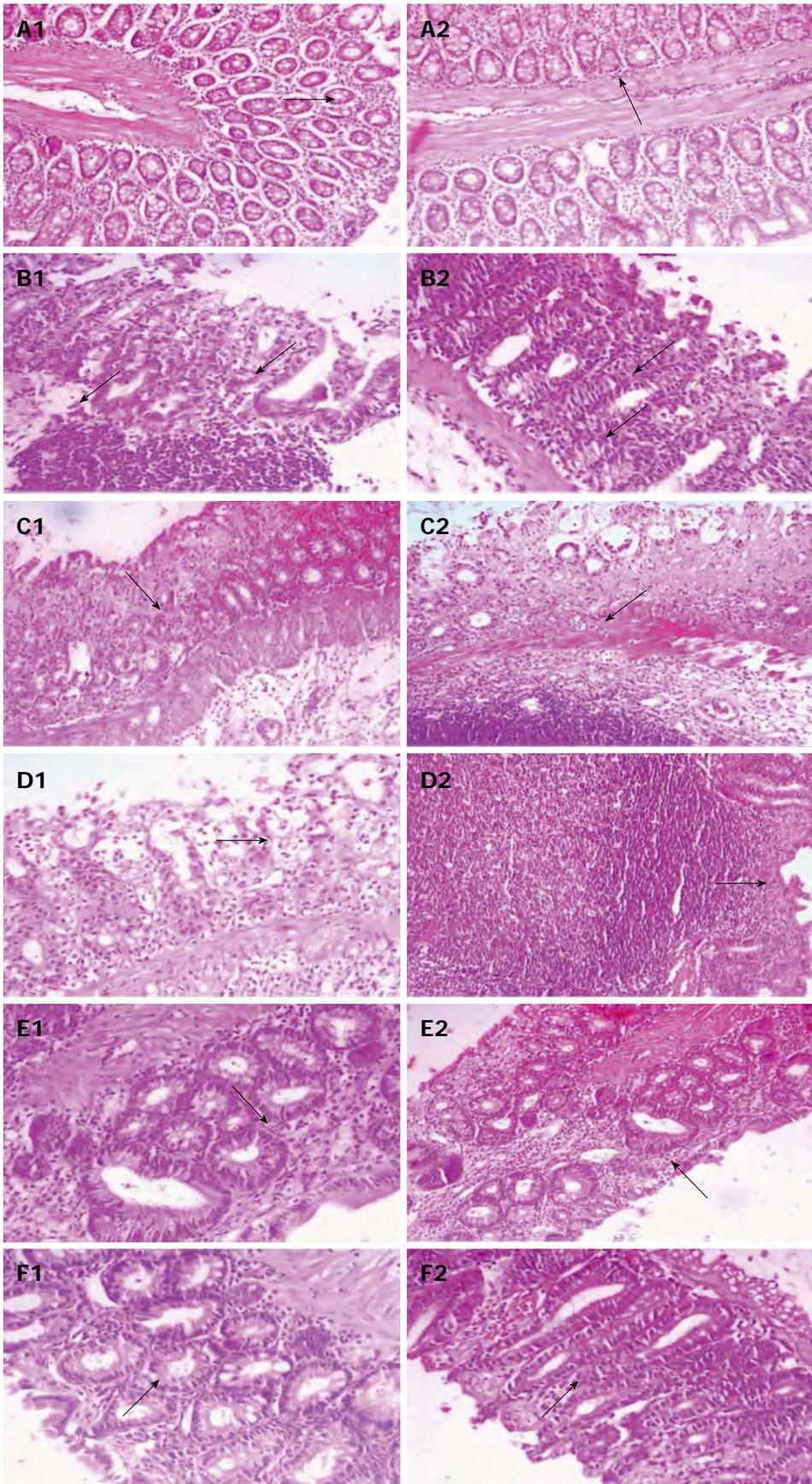


Figure 2 Histopathological changes with their intensity are presented. A-1 and 2: Histopathological colonic sections showing normal benign looking mucosa; B-1 and 2: Diffused active colitis with superficial erosions, stromal edema, dense acute and chronic inflammatory cells infiltrate with widely ulcerating mucosa; C-1 and 2: Reparative epithelial changes with little ulcer healing and inflammatory cells infiltrate; D-1 and 2: Reparative epithelial changes and healing ulcer with lymphoid follicle form; E-1 and 2: Healing ulcer and reparative epithelial changes; F-1 and 2: Attenuated cell damage with complete ulcer healing. A1-F1 ( $\times 400$ ), A2-F2 ( $\times 200$ ).

**Table 1** Effect of naringenin on microscopic scoring of histopathological sections of colonic tissue of rats with acetic acid-induced ulcerative colitis

Groups	Ulceration	Hyperemia	Necrosis	Edema	Cellular infiltrate	Goblet cell hyperplasia
Control	0	0	0	0	0	0
AA	+++	+++	++++	+++	++++	++
NG25 + AA	++	++	+++	++	+++	++
NG50 + AA	++	++	++	++	+++	+
NG100 + AA	+	+	+	+	++	+
MES + AA	+	+	+	+	+	+

MES: Mesalazine; AA: Acetic acid; NG: Naringenin; NG25: NG 25 mg/kg per day; NG50: 50 mg/kg per day; NG100: 100 mg/kg per day; 0: Normal; +: Mild; ++: Moderate; +++: Sever; ++++: Very sever.

hyperplasia and transmural lymphoplasmacytic infiltrate with few intraepithelial neutrophilic cells within stromal (Table 1 and Figure 2B). In NG25 + AA group, slight healing epithelial cells with scattered superficial ulcers lined by colonic glands with reparative epithelial changes with hyperchromatic nuclei and infrequent mitosis and less goblet cells surrounded by transmucosal fewer lymphoplasmacytic infiltrate within stromal edemas was seen (Table 1 and Figure 2C). Slide from NG50 + AA group showed intestinal rat lined by healing epithelial cells with tall columnar epithelium, with superficial shredded epithelial cells, less eroded surface surrounded by few inflammatory edema and less necrosis with colonic gland showed reparative epithelial changes (Table 1 and Figure 2D). In higher dose of NG treatment (NG100 + AA) group, superficial tiny eroded mucosa with mucosal, hemorrhage, edema and scattered acute and chronic inflammatory cells infiltrate surrounding colonic glands with reparative epithelial changes and few goblet cells were seen (Table 1 and Figure 2E). Slide from MES + AA group revealed intestinal section with more better healed and improvement of intestinal mucosa compared to positive controlled sections with few mucosal lymphoplasmacytic infiltrate within stromal edema (Table 1 and Figure 2F).

Acetic acid administration resulted in a significant ( $P < 0.01$ ) decrease in colon levels of both T-GSH and NP-SH when compared to control animals. Pretreatment with NG with higher doses (50 mg/kg and 100 mg/kg) significantly ( $P < 0.01$ ) attenuated T-GSH and NP-SH ( $P < 0.05$ ) the reduced levels as compared to AA group. Pretreatment with MES significantly inhibited the decreased levels of T-GSH and NP-SH ( $P < 0.001$  and  $P < 0.05$ , respectively) (Figure 3A and B). Concentration of TBARS in the colons of AA treated rats were significantly ( $P < 0.01$ ) increased compared to control animals. A significantly lower concentrations of TBARS values were found in NG25 + AA ( $P < 0.05$ ), NG50 + AA ( $P < 0.01$ ), NG100 + AA ( $P < 0.01$ ) and MES + AA ( $P < 0.01$ ) groups as compared to AA group (Figure 3C). CAT activity was significantly ( $P < 0.01$ ) decreased in colon tissues of AA administered rats compared to control animals. Pretreatment with 50 mg/kg per day and 100 mg/kg per day of NG, significantly ( $P < 0.05$ ) inhibited the decrease CAT activity in colon as compared to AA group (Figure 3D). SOD activity was significantly ( $P < 0.01$ ) reduced in the colons of AA treated animals as compared to control rats. Group

of rats pretreated with 100 mg/kg per day of NG for 7 d showed a significant ( $P < 0.05$ ) increase in colon SOD activity while compared to AA treated animals (Figure 3E). Pretreatment with MES also markedly ( $P < 0.05$  and  $P < 0.01$ , respectively) enhanced the CAT and SOD activities as compared to AA group (Figure 3D and E).

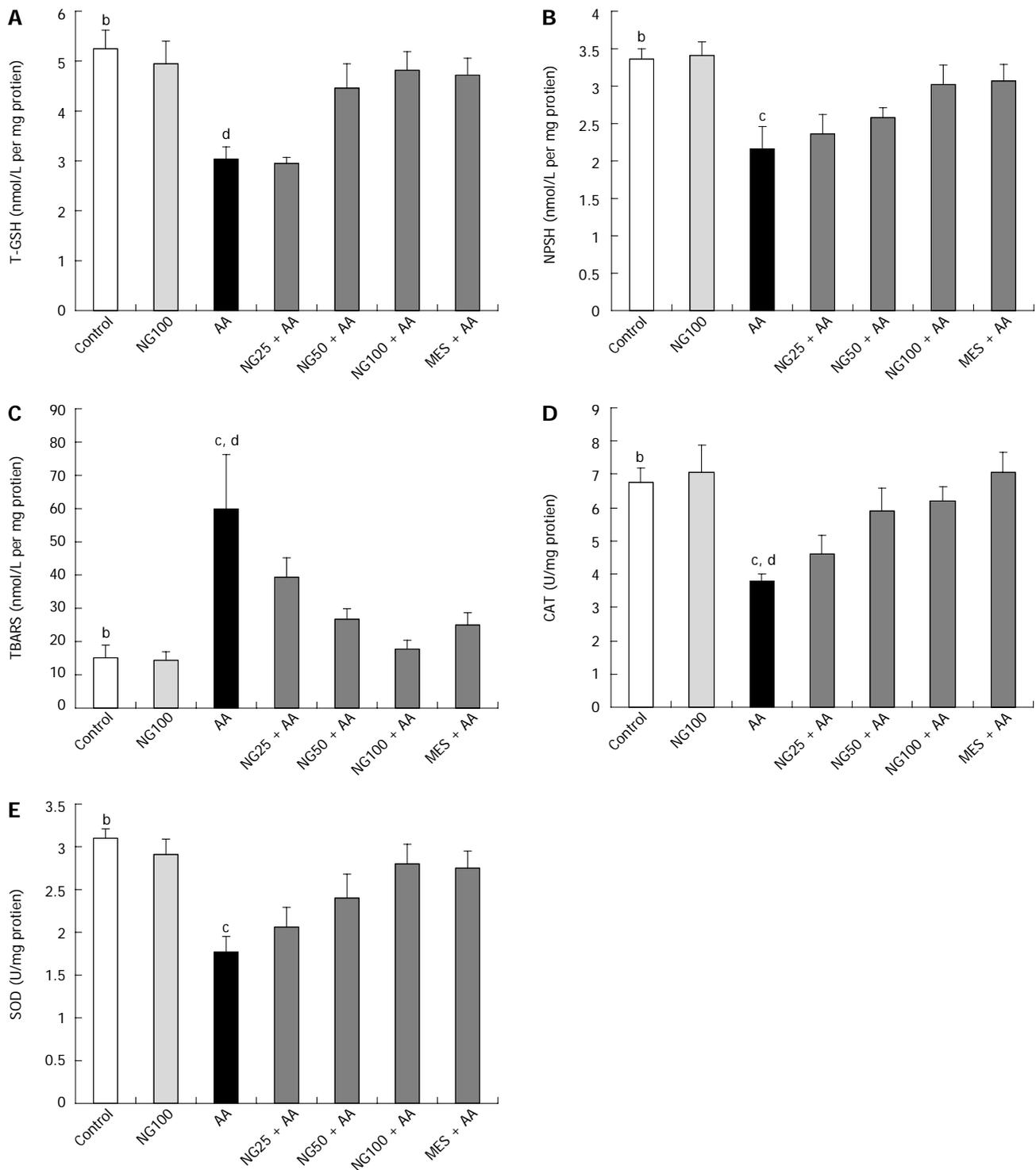
There was a significant ( $P < 0.001$ ) decrease in colon levels of DNA, RNA and total protein in AA administered group as compared to control animals. Pretreatment with NG (100 mg/kg per day) or MES (300 mg/kg per day) significantly ( $P < 0.01$ ) increased the DNA content in colon tissue compared to AA group. The RNA levels in NG higher doses and MES groups found significant ( $P < 0.01$ ) elevation compared to AA group. Total protein levels were also significantly ( $P < 0.05$ ) increased in NG50 + AA, NG100 + AA and MES + AA groups compared to AA group (Table 2).

Pro-inflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels produced significant ( $P < 0.01$ ) increase in AA-induced ulcerative colitis and levels found significantly ( $P < 0.05$  and  $P < 0.01$ ) diminished in NG higher doses and MES pretreated groups as compared to AA group, respectively (Figure 4A-C). Similar changes in PGE<sub>2</sub> levels were seen in colon tissue of rats (Figure 4D). In colon tissue, NO levels were significantly ( $P < 0.01$ ) increased AA group compared to controls. The elevated NO levels were markedly ( $P < 0.05$ ) reduced in NG and MES treated group compared AA group respectively (Figure 4E).

## DISCUSSION

Present investigation outlines the anti-ulcerogenic effect of NG against experimentally induced UC in rats as a model for IBD. The preventative effect of NG was confirmed by histological evaluation and also using MES as a standard drug. Seven days pretreatment with NG significantly reduced the AA-induced colonic mucus content and prevented oxidative and inflammatory response in dose dependent manner.

Ulcerative colitis is characterized by mucosal inflammation and ulcerations with a variable extent and severity<sup>[34]</sup>. Rectal administration of 4% AA to experimental rodents to induce UC is a well-established animal model, which phenotypically resembles human colon inflammation<sup>[35]</sup>. It also causes colonic epithelial lesions and



**Figure 3** Effect of naringenin on total glutathione sulphadryls (A), non-protein sulphadryls (B) and thiobarbituric acid reactive substances levels (C) as well as catalase (D) and superoxide dismutase activities (E) in colonic tissue of rats with acetic acid-induced ulcerative colitis ( $n = 6$ ). Values are expressed as mean  $\pm$  SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. <sup>b</sup> $P < 0.01$  control vs AA; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  AA vs NG25 + AA, NG50 + AA, NG100 + AA or MES + AA groups. T-GSH: Total glutathione sulphadryls; NP-SH: Non-protein sulphadryls; TBARS: Thiobarbituric acid reactive substances; CAT: Catalase; SOD: Superoxide dismutase; AA: Acetic acid; UC: Ulcerative colitis; MES: Mesalazine; NG: Naringenin.

necrosis associated with neutrophils and macrophages infiltration to the damaged colon indicating inflammatory conditions<sup>[28,35]</sup>. In present study, the 4% AA administration resulted a significant increase in colonic weight and induced sever ulceration and tissue necrosis associated with inflammatory infiltrate and goblet cell hyperplasia

as indicated in the results of the histopathological estimations. Similar pathological impairments were reported in earlier studies using the same animal model<sup>[26,36]</sup>. Application of AA in the present study disturbed the colonic mucus, which is in agreement with Popov *et al*<sup>[28]</sup>. Colonic mucus plays an important protective role against

**Table 2** Effect of naringenin on DNA, RNA and total protein levels in colonic tissue of rats with acetic acid-induced ulcerative colitis

Groups	DNA ( $\mu\text{g}/100$ mg wet tissue)	RNA ( $\mu\text{g}/100$ mg wet tissue)	Total protein (mg/100 mg wet tissue)
Control	652.05 $\pm$ 17.12 <sup>b</sup>	378.51 $\pm$ 38.44 <sup>b</sup>	3.0 $\pm$ 0.19 <sup>b</sup>
NG100	688.84 $\pm$ 47.69	380.24 $\pm$ 48.96	2.43 $\pm$ 0.31
AA	222.69 $\pm$ 18.78 <sup>d</sup>	167.35 $\pm$ 15.16 <sup>d</sup>	0.95 $\pm$ 0.08 <sup>e</sup>
NG25 + AA	293.93 $\pm$ 33.49	208.68 $\pm$ 25.05	1.32 $\pm$ 0.13
NG50 + AA	338.54 $\pm$ 21.44	250.06 $\pm$ 10.11	2.02 $\pm$ 0.46
NG100 + AA	415.50 $\pm$ 41.51	298.34 $\pm$ 12.92	2.15 $\pm$ 0.27
MES + AA	425.04 $\pm$ 38.23	307.44 $\pm$ 18.31	2.14 $\pm$ 0.26

Values are expressed as mean  $\pm$  SE ( $n = 6$ ) and analyzed using one way ANOVA followed by Newman-Keuls *post hoc* test. <sup>b</sup> $P < 0.01$  control *vs* acetic acid (AA); <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  AA *vs* Naringenin (NG) 25 + AA, NG50 + AA, NG100 + AA or mesalazine (MES) + AA groups.

chemically induced ulceration which may also facilitate the repair of the damaged epithelium<sup>[37]</sup>. Although, numerous pharmacotherapies have been suggested for UC treatment, the side effects or toxicity of these medications are a major clinical problem<sup>[38]</sup>. That is why naturally occurring products such as flavonoids are now suggested as an alternative option beside the conventional therapies<sup>[39]</sup>. Indeed, earlier experimental studies demonstrated flavonoids such as quercitrin, kushenin, kaempferol and baicalin to promote UC healing<sup>[40-43]</sup>.

Previous studies demonstrated that NG administration effectively protected the experimentally induced gastric lesions and ulcers<sup>[44,45]</sup>. Protection against experimental UC induced by NG was accompanied by restoration of the increased colon thickening in AA group, which is an indirect assessment of colon inflammation. Microscopic scoring of the histopathological sections confirmed the protective action of NG as it decreased colonic tissue ulceration, necrosis and inflammation in dose dependent manner. Motilva *et al.*<sup>[45]</sup> reported that NG treatment increased the gastric mucus levels in rats induced gastric lesions by absolute ethanol. In the present study, NG was also found to inhibit the depletion of colonic mucus caused by AA treatment. This protective activity could be attributed to its antioxidant and anti-inflammatory properties.

Oxidative stress is known to play an important role in IBD initiation and progression<sup>[46]</sup>. Experimentally induced colitis in animals is characterized by oxidative damage and an imbalance between oxidant and antioxidant substances<sup>[47]</sup>. The AA-induced colitis model is known to cause vascular dilatation and white blood cells accumulation, as well as an increase in blood flow, leading to increased production of oxygen and hence the excessive generation of free radical and ROS<sup>[35,48]</sup>. Several studies have indicated the vital role that free radicals play in the pathogenesis of mucosal injuries<sup>[49,50]</sup>. Moreover, free radicals and ROS were reported in colorectal specimens of ulcerative colitis<sup>[51,52]</sup>. The first line of oxidative defense system against free radicals is the sulphadryls groups in peptide namely GSH or NP-SH. It is widely distributed in all biological

tissues and work as a non-enzymatic antioxidant. GSH inhibits ROS oxidative injuries directly *via* its sulfhydryl group and indirectly as a cofactor or a coenzyme in ROS enzymatic detoxification process<sup>[53,54]</sup>. Another line in oxidative defense system is the enzymatic antioxidants. Examples for important antioxidant enzymes are SOD, CAT, and GPx<sup>[55]</sup>. In present study, levels and activities of non-enzymatic and enzymatic defense systems were severely decreased in the colon of AA administered animals indicating oxidative cellular injury. Furthermore, free radicals are known to attack lipid contents of cellular membranes leading to activation of LPO process and cellular damage. Therefore, the concentrations of LPO specific products such as TBARS were elevated, while the critical cellular macro- and micro-molecules such as nucleic acids and total proteins levels were decreased in the present work indicating cellular oxidative injury and cytotoxicity. These results, which are in agreement with previous findings, suggest the harmful effects of AA on cellular macromolecules and its ability to impair the epithelial cell integrity and hinder mucosal recovery<sup>[23,36]</sup>.

In present study, NG was able to attenuate AA induced oxidative damage and injury of colon tissues confirming its strong antioxidant and anti-inflammatory properties. It has seen in earlier studies that NG markedly increased the antioxidant markers such as GSH, NP-SH levels and SOD, and CAT activities<sup>[12,56,57]</sup>. Han *et al.*<sup>[56]</sup> found that NG pretreatment can increase the activity of antioxidant enzyme GPx, which suggest the ability of NG to attenuate oxidative stress by decreasing the lipid peroxide level and to inhibit accumulation of free radicals generation during LPO process<sup>[57]</sup>. In the current study, NG treatment significantly corrected the impaired levels of nucleic acids and total protein in colon tissue suggesting the cytoprotective properties of the naturally occurring flavonoids. The antioxidant activity of NG depends mainly on the presence of B-ring catechol group, which can stabilize a radical species by donating hydrogen ( $\text{H}^+$ )<sup>[12]</sup>.

Inflammatory cytokines are known to play a crucial role in modulating mucosal immune system where the neutrophils and macrophages are responsible for disrupting epithelial integrity and causing colon injury<sup>[58]</sup>. The pathogenesis of UC is characterized by migration of granulocytes and other leukocytes to the inflamed mucosa and superficial ulcers leading to increased levels of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$ <sup>[59,60]</sup>. In present study, the elevated colon level of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in AA administered group is an evidence for epithelial cell necrosis, edema, and neutrophil infiltration, which is also supported by the histopathological results. These results are in accordance with earlier experimental and clinical studies<sup>[6,28,36,61]</sup>. The reported increased levels of colonic PGE<sub>2</sub> in AA group of animals is in agreement with Otani *et al.*<sup>[62]</sup>, where the enhanced level of PGE<sub>2</sub> was attributed to its overproduction rather than decreased metabolism, both of which are mediated by pro-inflammatory cytokines. Naringenin was found in the current

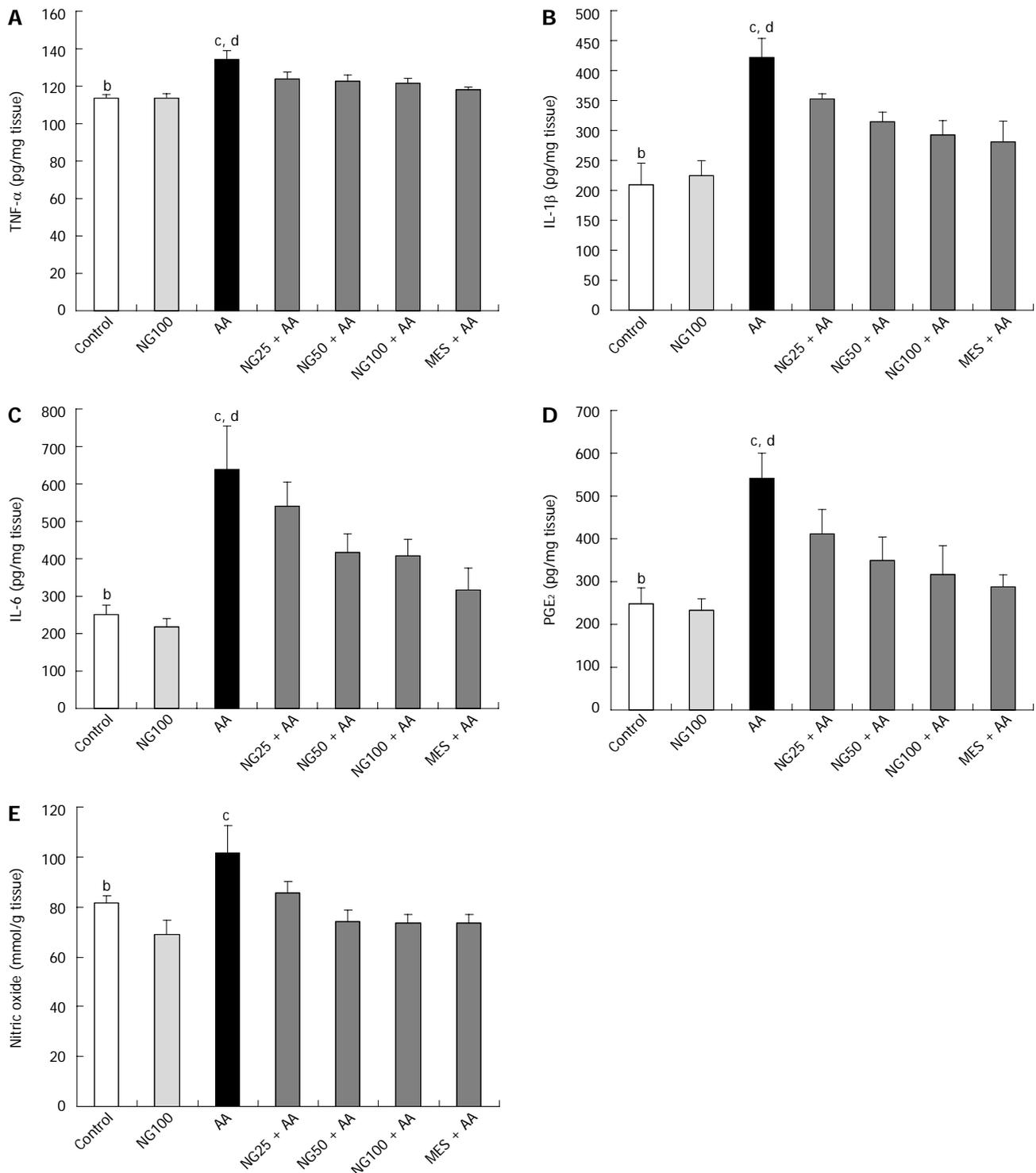


Figure 4 Effect of naringenin on tumor necrosis factor- $\alpha$  (A), interleukin-1 $\beta$  (B), interleukin-6 (C), prostaglandin E $_2$  (D) and nitric oxide (E) levels in colonic tissue of rats with acetic acid-induced ulcerative colitis ( $n = 6$ ). Values are expressed as mean  $\pm$  SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. <sup>b</sup> $P < 0.01$  control vs AA; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  AA vs NG25 + AA, NG50 + AA, NG100 + AA or MES + AA groups. TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ : Interleukin-1 $\beta$ ; IL-6: Interleukin-6; PGE $_2$ : Prostaglandin E $_2$ ; AA: Acetic acid; NG: Naringenin; MES: Mesalazine.

and earlier studies to inhibit the level of inflammatory cytokines including TNF- $\alpha$ , IL-6 and IL-1 $\beta$ <sup>[63]</sup>. The anti-inflammatory properties of naringenin were suggested to be through several mechanisms including increased phosphorylation of ERK 5 and P38 MAPK and inhibition of NF- $\kappa$ B and activator protein-1 signaling<sup>[64,65]</sup>. Additionally, naringenin, which present in high concentrations in cit-

rus fruits, was found to block NF- $\kappa$ B activation resulting in down regulation of the downstream target genes of NF- $\kappa$ B such as iNOS and COX-2 expression<sup>[66]</sup>. These enzymes catalyze oxidative stress-induced production of NO and prostaglandins respectively, which are known as an important inflammatory mediators in the pathogenesis of colitis<sup>[63,67]</sup>. These findings are in agreement with our

results where pretreatment with naringenin significantly ameliorated AA induced elevation of the level of PGE<sub>2</sub> and NO in rats' colon.

In conclusion, the present study revealed that NG-protects the AA-induced ulcerative colitis by inhibiting inflammatory and oxidative bio-markers. Finally, our results may pose promising outcomes for future clinical usage of NG as a natural non-toxic effective supplement in IBD.

## COMMENTS

### Background

The pathogenesis of inflammatory bowel disease (IBD) such as ulcerative colitis (UC) is usually associated with reduced antioxidant capacity. Generation of free radicals like reactive oxygen species (ROS) leads to lipid peroxidation, which inhibits cellular antioxidant capability, resulting in prominent colonic inflammation. There is a great need to search for safe and tolerable compounds for the management of IBD to reduce patient compliance as well as the adverse effects of conventional treatments. Naringenin (NG) is a naturally occurring flavonoid that can be extracted from citrus fruits, tomatoes, cherries, grapefruit, and cocoa. Like most of the flavonoids, NG was experimentally found to have several pharmacological potentials, including antioxidant, antitumor and anti-inflammatory because of NG has properties to produce sufficient hydroxyl (-OH) substitutions, which give it the capability to scavenge ROS. Thus, it has considered that NG may diminish and/or improve pathological conditions where oxidation or inflammation is deemed to play a vital role, like in case of IBD.

### Research frontiers

In the present study, NG was orally (gavage) treated with three doses (25, 50 and 100 mg/kg per day body weight) for 7 consecutive days, 24 h later UC was induced by 4% acetic acid. In colitis tissue, Alcian blue, pro-oxidative and inflammatory biomarkers were estimated. The biochemical alterations were further justified with histopathological changes.

### Innovations and breakthroughs

NG pretreatment clearly revealed the protection against acetic acid-induced UC in animal model. Antioxidant and anti-inflammatory properties of NG are suggested to be the key for these effects as NG significantly reduced oxidative stress and inflammatory biomarkers in a dose dependent manner.

### Applications

The present data shows that NG has a promising protective effect against experimentally-induced UC in animal model. Thus, NG could be recommended for its use as potential alternative and complementary therapy for IBD after confirmation of the obtained findings by clinical trials.

### Peer review

The preclinical preventative properties of NG against UC are outlined in present study. Also the possible pharmacological mechanisms of action responsible for these effects are evaluated. Overall, this study proofed that NG is an effective and safe compound that worth to be investigated in future clinical trials for its colonic anti-ulcerogenic properties.

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## Quality of compounded topical 2% diltiazem hydrochloride formulations for anal fissure

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### Abstract

**AIM:** To investigate the quality of topical 2% diltiazem formulations extemporaneously compounded by retail pharmacies openly offering drug-compounding services.

**METHODS:** A participating healthcare professional wrote 12 prescriptions for compounded 2% diltiazem cream, with 2 refills allowed per prescription. The 12 sets of prescriptions were filled, at intervals of 1-2 wk between refills, at 12 different independent retail pharmacies that openly offer drug-compounding services in a major metropolitan region. The 36 resultant preparations, provided as jars or tubes, were shipped, as soon as each was filled, at ambient temperature to the study core laboratory for high-performance liquid chromatography (HPLC) analysis, within 10 d of receipt. For the HPLC analysis, 8 different samples of the topical diltiazem, each approximately 1 g in weight, were taken from prespecified locations within each container. To initiate the HPLC analysis, each sample was transferred

to a 100 mL volumetric flask, to which methanol was added. The HPLC analysis was conducted in accordance with the laboratory-validated method for diltiazem in cream, ointment, and gel formulations. The main outcome measures were potency (percentage of label claim) and content uniformity of the compounded topical 2% diltiazem formulations.

**RESULTS:** Of the 36 prescriptions filled, 30 were packaged in jars and 6 were packaged as tubes. The prescriptions were specifically for cream formulations, but 6 of the 12 pharmacies compounded 2% diltiazem as an ointment; for another pharmacy, which had inadequate labeling, the dosage form was unknown. The United States Pharmacopoeia (USP) standard for potency is 90%-115% of label claim. Of the 36 preparations, 5 (13.89%) were suprapotent and 13 (36.11%) were subpotent. The suprapotent prescriptions ranged in potency from 117.2% to 128.5% of label claim, and the subpotent prescriptions ranged in potency from 34.8% to 89.8% of label claim. Fourteen (38.9%) preparations lacked content uniformity according to the USP standard of 90%-110% potency and < 6% relative standard deviation. Of the 30 formulations packaged in jars, 12 (40%) lacked content uniformity, while of the 6 formulations packaged in tubes, 2 (33.3%) lacked content uniformity. Nine of the 12 pharmacies (75%) failed USP potency or content-uniformity specifications for at least 1 of the 3 prescription fills. For 5 of the 12 pharmacies (41.7%), the mean potency across all three prescription fills was < 90% of label claim.

**CONCLUSION:** Patients prescribed topical 2% diltiazem for treatment of anal fissure frequently receive compounded formulations that are misbranded with respect to potency and that lack content uniformity.

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**Key words:** Anal fissure; Pharmacy compounding; Topical diltiazem; Formulation potency; Content uniformity

**Core tip:** The use of topical 2% diltiazem hydrochloride for treating anal fissures is supported by multiple clinical trials and is recommended in published practice parameters. As no commercially manufactured formulation of topical 2% diltiazem has been approved yet by the Food and Drug Administration for the treatment of anal fissure, prescriptions for the medication need to be extemporaneously compounded by retail pharmacies. Employing high-performance liquid chromatography analysis of topical 2% diltiazem formulations compounded by a sampling of pharmacies, we found a notable trend toward lack of content uniformity and misbranding of potency, suggesting that many patients might not receive the anticipated relief of anal-fissure pain.

Shah M, Sandler L, Rai V, Sharma C, Raghavan L. Quality of compounded topical 2% diltiazem hydrochloride formulations for anal fissure. *World J Gastroenterol* 2013; 19(34): 5645-5650 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5645.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5645>

## INTRODUCTION

The use of topical 2% diltiazem hydrochloride for treating anal fissures by lowering anal sphincter pressure has been explored in multiple clinical trials since 2000<sup>[1-5]</sup>. Diltiazem hydrochloride, a calcium channel blocker, is well known as an oral treatment for hypertension and angina<sup>[6]</sup>. In 2010, the Standard Practice Task Force of the American Society of Colon and Rectal Surgeons (ASCRS) published revised practice parameters for managing anal fissure, assigning to each practice parameter a grade of recommendation and a class of evidence<sup>[7]</sup>. Noting that conservative (nonsurgical) therapy is safe and should be the first step for managing anal fissure<sup>[8-10]</sup>, the ASCRS task force stated that topical formulations of calcium channel blockers may be appropriately used to treat anal fissure and that these drugs seemed to have a lower incidence of adverse effects than topical nitrates, such as nitroglycerin. This practice parameter was accorded the highest grade of recommendation and the second highest class of evidence by the task force<sup>[7]</sup>.

No commercially manufactured formulation of topical 2% diltiazem has been approved by the United States Food and Drug Administration (FDA) for the treatment of anal fissure. Consequently, colon and rectal surgeons, gastroenterologists, and other physicians who want to follow ASCRS practice parameters and prescribe a topical calcium channel blocker for treatment of anal fissure have to write prescriptions for a product that will be extemporaneously compounded by retail pharmacies. Directions for compounding 2% diltiazem as a topical formulation are readily available in published literature. For example, propylene glycol, hydroxyethyl cellulose, and heated purified water are mixed with diltiazem, and

the resulting formulation is packaged in a tight, light-resistant container, usually a tube or jar<sup>[11]</sup>. A few pharmacies that specialize in compounding services advertise the availability of compounded topical 2% diltiazem on the internet. However, many nonspecialized retail pharmacies also fulfill prescriptions by compounding the product.

In 2006, the FDA investigated the quality of compounded products, collecting active pharmaceutical ingredients (API) and finished compounded drug samples during unannounced visits to compounding pharmacies throughout the country. All API samples passed analysis, but a third of the 36 compounded samples that were collected failed analysis by being either subpotent or suprapotent or by lacking content uniformity. The United States Pharmacopoeia (USP) standard for potency is 90%-115% of label claim<sup>[12]</sup>. Because the API samples passed analysis, the FDA observed that the failures of the samples in the analysis were directly related to faulty compounding processes at the pharmacies, including the lack of proper in-process controls and end-product testing<sup>[13]</sup>.

To examine the quality of compounded formulations of topical 2% diltiazem, we undertook a high-performance liquid chromatography (HPLC) analysis of preparations gathered from retail pharmacies in a major metropolitan region.

## MATERIALS AND METHODS

### Data source

A healthcare professional was asked to write prescriptions for extemporaneously compounded 2% diltiazem cream for fulfillment by retail pharmacies in the greater New York metropolitan region. The selection criteria, intended to locate retail pharmacies that might have experienced pharmacists on staff with competency at compounding, included stipulations that the pharmacies be independent, not parts of retail pharmacy chains, and that they openly offer drug-compounding services by means of online or other advertising. A total of 12 qualifying retail pharmacies were selected from different parts of the metropolitan region.

The participating healthcare professional wrote 12 prescriptions, with 2 refills allowed per prescription, so that 3 prescriptions could be filled at each of the 12 pharmacies (36 total fills) for compounded 2% diltiazem cream. The prescriptions were filled at each of the 12 pharmacies during May 2012 and June 2012, at intervals of 1-2 wk in between refills. As soon as any prescription was filled by a retail pharmacy, it was collected and shipped at ambient temperature in a prepared bubble-wrap mailer to DermPathe Pharmaceuticals (Branchberg, NJ, United States), the laboratory engaged for the HPLC analysis. The compounded formulations of topical 2% diltiazem were provided by the retail pharmacies as either jars or tubes.

Upon receipt of each package, DermPathe logged the time and date and stored the compounded formulation at ambient temperature. HPLC analysis was conducted

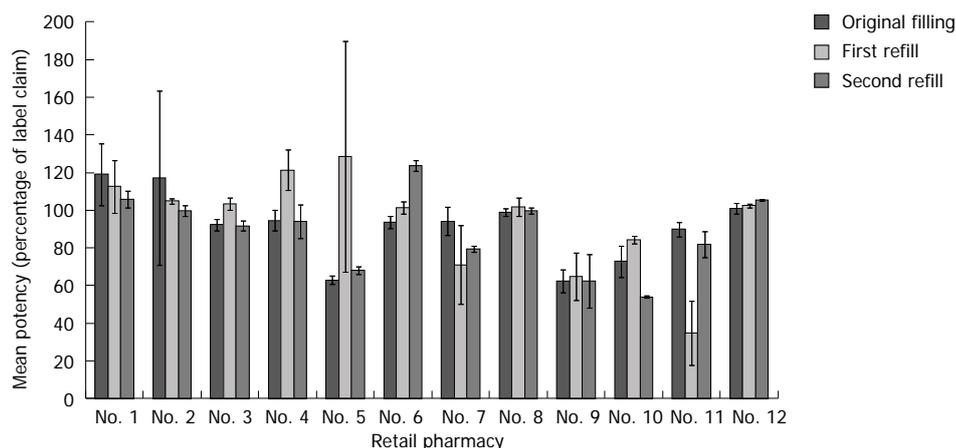


Figure 1 Mean potency of diltiazem content in each of the 3 samples from each of the 12 different retail pharmacies (mean  $\pm$  SD).

in accordance with the DermPathe-validated method for diltiazem in cream, ointment, and gel formulations. The analysis included an assessment of potency (percentage of label claim) and content uniformity for each of the compounded formulations. For the analysis of each formulation, 8 different samples of the topical diltiazem, each approximately 1 g in weight, were taken from pre-specified locations within the compounded formulation container. If the compounded formulation was provided in a jar, 4 samples were taken from the top of the jar: 1 at the top front, 1 at the top center, 1 at the top right back corner, and 1 at the top left back corner. In the middle layer of the jar, 1 sample was taken from the center and 1 from the middle right corner. At the bottom of the jar, 1 sample was taken from the center and 1 from the bottom left corner. If the compounded formulation was provided in a tube, the tube was sectioned horizontally and opened up. Then 2 samples were drawn from the top left and top right of the tube, 2 more from the middle left and middle right of the tube, 2 from the bottom left and bottom right of the tube, and the final 2 randomly on the left and on the right between the middle and the bottom of the tube.

Each sample was transferred to a 100 mL volumetric flask, to which methanol was added to fill approximately 80% of the flask volume. The solution was sonicated for 1 h, and the flask was then filled to volume with methanol and mixed thoroughly by shaking. Filtered through a 0.45-micron filter, the solution was then transferred into HPLC vials for analysis using a Waters 2695 HPLC system with a 2487 dual wavelength detector (Waters Corporation, Milford, MA, United States). A Luna C8 150 mm  $\times$  4.6 mm, 5 mm column with a C8 Security Guard column (Phenomenex, Torrance, CA, United States) was used as the stationary phase. The mobile phase consisted of an acetate buffer, acetonitrile, and methanol, in a 50:25:25 ratio. The acetate buffer contained 8.2 g of anhydrous sodium acetate and 1.16 g of *D*-10-camphorsulphonic acid in 1000 mL of water, with pH adjusted to 6.2 with 1 mol/L sodium hydroxide. The flow rate was set at 2 mL per minute, the detector wavelength was set at 240 nm, and the column temperature was set to 30 °C.

### Statistical analysis

Descriptive statistics were used in this study. For categorical variables, frequencies and percentages are reported. For continuous variables, the number of observations, mean  $\pm$  SD, and relative standard deviation are reported. Statistical analyses were performed using Excel 2010 (Microsoft, Redmond, WA, United States).

## RESULTS

Thirty-six prescriptions for compounded topical 2% diltiazem were written, filled, and shipped to DermPathe for analysis. Of these preparations, 30 were packaged in jars and 6 were packaged as tubes (only 2 of the 12 retail pharmacies used tubes for packing). One of the 12 retail pharmacies failed to label each of the 3 filled prescriptions of topical 2% diltiazem with the drug name; the label on each of the 3 formulations from this pharmacy simply read “compound.”

The prescriptions were specifically for cream formulations. Five of the 12 pharmacies compounded 2% diltiazem as a cream, using lipoderm, but 6 of the 12 pharmacies compounded 2% diltiazem as an ointment, using petrolatum. For 1 pharmacy, the same that had the inadequate labeling, the dosage form was unknown.

The preparations were analyzed by DermPathe within 10 d of receipt. Prior to being analyzed, the products were stored at room temperature. In the published directions for preparing topical 2% diltiazem, the shelf life of the preparation is given as 30 d when stored at room temperature<sup>[11]</sup>. At the time of HPLC analysis, there were no visible signs of product degradation in any of the jars or tubes.

### Potency results

Of the 36 prescriptions, 18 (50.0%) were misbranded for potency according to the USP standard. Five (13.9%) of the prescriptions were suprapotent (that is, the measured drug activity was  $>$  115% of label claim) (Figure 1). The suprapotent prescriptions ranged in potency from 117.2% to 128.5% of label claim. No retail pharmacy produced more than 1 suprapotent formulation. Thirteen

**Table 1 Potency at prespecified locations of compounded preparations of topical 2% diltiazem that lacked content uniformity**

	Percentage of label claim (prescription number, type of packaging)													
Sample location <sup>1</sup>	1, jar	2, jar	4, jar	10, jar	11, jar	14, jar	19, jar	20, jar	25, jar	26, jar	27, jar	28, jar	32, tube	33, tube
Location 1	111.9%	110.8%	102.9%	97.5%	126.0%	83.3%	100.2%	71.4%	73.0%	39.9%	94.8%	83.8%	44.3%	67.4%
Location 2	108.7%	114.2%	95.7%	98.0%	117.8%	73.1%	99.3%	20.9%	58.7%	74.2%	68.9%	66.5%	15.1%	83.4%
Location 3	142.1%	112.0%	100.6%	101.1%	144.9%	93.4%	91.2%	82.3%	67.3%	52.1%	63.4%	81.1%	16.8%	80.7%
Location 4	149.1%	145.5%	99.8%	100.9%	117.1%	72.4%	99.9%	81.8%	64.9%	76.1%	57.7%	82.9%	33.8%	87.9%
Location 5	110.6%	106.5%	109.0%	92.9%	107.4%	112.4%	95.9%	69.2%	63.2%	67.9%	54.3%	71.5%	67.7%	81.5%
Location 6	106.7%	99.9%	97.1%	90.9%	116.1%	161.1%	97.3%	81.3%	59.5%	65.8%	51.5%	65.4%	29.4%	79.3%
Location 7	110.8%	103.8%	100.8%	85.9%	121.2%	211.2%	77.8%	79.9%	55.2%	72.0%	56.1%	65.6%	42.0%	83.0%
Location 8	111.7%	107.8%	231.7%	89.2%	120.6%	220.8%	91.0%	82.1%	57.6%	70.6%	52.8%	65.6%	29.4%	90.7%
Mean	119.0%	112.6%	117.2%	94.6%	121.4%	128.5%	94.1%	71.1%	62.4%	64.8%	62.4%	72.8%	34.8%	81.7%
Relative SD	14.0%	12.5%	39.6%	6.0%	9.0%	46.9%	8.0%	29.4%	9.4%	19.2%	22.9%	11.5%	48.5%	8.5%

<sup>1</sup>Jars: 1: Top front; 2: Top left back corner; 3: Top right back corner; 4: Top center; 5: Center of jar; 6: Middle right corner; 7: Bottom left corner; 8: Bottom center. Tubes: 1: Top left; 2: Top right; 3: Middle left; 4: Middle right; 5: Random left; 6: Random right; 7: Bottom left; 8: Bottom right.

(36.1%) of the prescriptions were subpotent (that is, the measured drug activity was < 90% of label claim) (Figure 1). The subpotent prescriptions ranged in potency from 34.8% to 89.8% of label claim. Only 3 of the 12 pharmacies compounded each of the 3 prescriptions they filled without misbranding potency. For 3 of the 12 pharmacies, all 3 of the filled prescriptions were subpotent.

### Content uniformity results

Of the 36 preparations, 14 (38.9%) lacked content uniformity according to the USP requirement of 90% to 110% potency and < 6% relative standard deviation<sup>[12]</sup>. Table 1 shows the potency variations at the different sample locations of these 14 preparations. Of the 30 formulations packaged in jars, 12 (40%) lacked content uniformity; of the 6 formulations packaged in tubes, 2 (33.3%) lacked content uniformity. In some of the jars the potency varied by more than 100%. In batch 4, provided as a jar, the potency at the top center of the jar was 99.8% while the potency at the bottom center of the jar was 231.7%. In batch 14, also provided as a jar, the potency at the top center of the jar was 72.4% while the potency at the bottom of the jar was 220.8%. Batch 32, provided as a tube, was overall subpotent and also lacked content uniformity: at the middle left of the tube, potency was 16.8%; at the bottom left of the tube, potency was 42.0%.

### Pharmacy performance

Nine of the 12 pharmacies failed USP potency or content-uniformity specifications for at least 1 of their 3 prescriptions. Three of the 12 pharmacies failed USP potency or content-uniformity specifications for all 3 of their prescriptions. When the potencies of the 3 time-separated prescriptions were averaged together for each of the 12 pharmacies, the mean potency was < 90% of label claim for 5 of the 12 pharmacies (Table 2).

## DISCUSSION

In this HPLC analysis of 36 preparations of compounded topical 2% diltiazem from 12 retail pharmacies, half

of the preparations did not meet USP specifications for potency and almost 40% of the preparations did not meet USP specifications for content uniformity. Of the 12 retail pharmacies, only three were able to fill all three of the time-separated prescriptions consistently within USP specifications.

When compounded preparations of topical 2% diltiazem fall outside USP specifications for potency, they are more likely to be subpotent (36.1% of the prescriptions) than suprapotent (13.9% of prescriptions). With more than a third of the prescriptions of compounded 2% diltiazem being subpotent, such prescriptions might not routinely relieve anal fissure pain to the extent or with the speed established in clinical trials of topical 2% diltiazem<sup>[1,4,14-16]</sup>. In one of those trials, Carapeti *et al*<sup>[1]</sup> compared different diltiazem gel concentrations (0.1%, 0.5%, 1%, 2%, 5%, and 10% weight per volume) and found a dose-dependent effect on maximum resting anal sphincter pressure (MRP), with the maximal effect (28% reduction compared with pretreatment,  $P < 0.0001$ ) achieved with the 2% formulation. The MRP was not lowered as effectively with the 1% concentration, while concentrations higher than the 2% produced no additional effect.

The potency of the 5 suprapotent preparations of topical 2% diltiazem did not exceed 128.5% of label claim. However, owing to a lack of content uniformity in some preparations, especially when packaged in jars, compounded diltiazem could be more than twice as potent as the label claim in some sections of a container. The level of suprapotency in these sections, as high as 231.7% of label claim in a section of one jar, could put patients at potential risk for drug-related side effects. Because diltiazem is also used as a hypertensive agent, the largest risk associated with suprapotent topical 2% diltiazem might be dizziness or postural hypotension. In studies that have evaluated topical 2% diltiazem for treatment of anal fissure, the side effect profile has been mild, and the most frequent side effects have been headache or anal pruritus<sup>[5,17-20]</sup>. However, there have been reports of postural hypotension associated with the use of topical diltiazem<sup>[21]</sup>.

Azarnoff *et al*<sup>[12]</sup> conducted a similar HPLC analysis

**Table 2 Mean potency as percentage of label claim for 3 prescriptions of compounded topical 2% diltiazem filled by 12 retail pharmacies**

Pharmacy No.	Label claim (mean $\pm$ SD)	Relative SD
1	95.9% $\pm$ 9.8%	8.7
2	107.3% $\pm$ 16.5%	15.4
3	95.9% $\pm$ 2.2%	2.2
4	103.3% $\pm$ 5.1%	4.9
5	86.5% $\pm$ 20.1%	23.2
6	106.2% $\pm$ 1.8%	1.7
7	81.6% $\pm$ 6.5%	8.0
8	100.1% $\pm$ 2.0%	1.9
9	63.2% $\pm$ 3.8%	6.0
10	70.4% $\pm$ 3.2%	4.5
11	68.8% $\pm$ 6.5%	9.4
12	102.9% $\pm$ 0.8%	0.8

of compounded formulations of topical 0.3% nitroglycerin ointment for anal fissure. The investigators acquired 24 filled compounded prescriptions from 24 retail pharmacies across different geographic regions. They found that 7 (29.2%) of the 24 compounded formulations were subpotent and that 1 (4.8%) was suprapotent. Moreover, 5 (20.8%) of the 24 samples lacked content uniformity. In comparison, in the current study, in which 36 compounded preparations were acquired from 12 different pharmacies, 13 (36.11%) of the preparations were subpotent, 5 (13.9%) were suprapotent, and 14 (38.9%) lacked content uniformity. The relatively worse analytic performance of compounded topical 2% diltiazem formulations in the current study might be an artifact of study design. Another explanation might be that it is more difficult to prepare compounded diltiazem formulations rather than compounded nitroglycerin formulations in accordance with USP standards, although it is unclear why this might be the case.

In October 2012, an FDA report of an outbreak of fungal meningitis related to contaminated products produced by the New England Compounding Center (Framingham, MA, United States) brought to public awareness the practice and business of pharmacy compounding<sup>[22,23]</sup>. There are legitimate reasons for physicians to prescribe extemporaneously compounded drugs: for example, to provide patients with products like topical 2% diltiazem, currently recommended in the ASCR practice parameters for anal fissure but not approved by the FDA, or to create unique medications for specific patients, such as those who have documented allergies to certain drug ingredients or who require dosage forms different from those of FDA-approved drugs<sup>[24-26]</sup>. However, not only have both branded and generic drugs undergone FDA approval for safety and efficacy, they are also required by law to be produced under federal Good Manufacturing Practice (GMP) regulations in order to ensure their identity, quantity, potency, and purity<sup>[24]</sup>. In contrast, although extemporaneously compounded drugs might be formulated under professional pharmacy standards, these standards are inherently less rigorous than federal GMP quality standards. Because there is no federal surveillance

of compounded drugs, the extent of quality and safety problems with compound drugs is unknown<sup>[24]</sup>. Clearly, a topical 2% diltiazem cream produced under GMP regulations is needed to avoid the large percentage of substandard compounded formulations of a drug specifically recommended by the practice parameters of a medical society.

There were limitations to this study. The sample size was small, and the collection of samples was restricted to a single major metropolitan area. None of the compounded formulations were analyzed for microbiologic content when received by the laboratory. Upon routine inspection of the formulations after 8 mo of refrigerated storage at 5 °C, the laboratory discovered mold on some of them, and all samples were discarded. A future study of compounded formulations of topical 2% diltiazem will need to include analyses of microbiologic content, of possible drug degradation, and of the release of diltiazem from the formulations.

In conclusion, this study shows that when patients are prescribed topical 2% diltiazem cream for treatment of anal fissure in a major metropolitan region, they receive compounded formulations from retail pharmacies that are misbranded in respect to potency approximately 50% of the time and that lack content uniformity approximately 40% of the time. Because approximately one third of the compounded preparations were subpotent, patients treated with compounded formulations of topical 2% diltiazem might not receive the anticipated relief of pain associated with anal fissure.

## COMMENTS

### Background

The use of topical 2% diltiazem hydrochloride for treating anal fissures is supported by multiple clinical trials and is recommended in published medical society practice parameters. In countries where no commercially manufactured formulation of topical 2% diltiazem is available, prescriptions for the medication need to be extemporaneously compounded by retail pharmacies. Up to now, the quality of these compounded diltiazem formulations has not been evaluated.

### Research frontiers

High-performance liquid chromatography analysis was undertaken with 36 preparations of compounded 2% topical diltiazem that were gathered from 12 different independent retail pharmacies in a major metropolitan area.

### Innovations and breakthroughs

This is the first study to show that when patients are prescribed topical 2% diltiazem cream for treatment of anal fissure, they receive compounded formulations from retail pharmacies that are misbranded in respect to potency approximately 50% of the time and that lack content uniformity approximately 40% of the time. Because over one third of the compounded preparations were subpotent, patients treated with compounded formulations of topical 2% diltiazem might not receive the anticipated relief of anal-fissure pain.

### Applications

By demonstrating that a sample of retail pharmacies compound a large percentage of substandard formulations of topical 2% diltiazem, the study underscores that this drug, recommended for use by medical society practice parameters, should be produced under Good Manufacturing Practice regulations to ensure its identity, quantity, potency, and purity.

### Peer review

Local absorption of diltiazem depends on skin thickness and local inflammation. It is also proportional to the medication amount. This study is similar to a 2007 study concerning compounded formulations of nitroglycerin ointment for anal

fissure. The authors noticed a problem that is presumably unknown to gastroenterologists and surgeons: that compounded formulations of topical 2% diltiazem, recommended by the American Society of Colon and Rectal Surgeons for anal fissure therapy but not approved by the United States Food and Drug Administration, may be subpotent or suprapotent. Of 36 compounded preparations examined in the study, 38.9% lacked content uniformity, and 50% did not meet United States Pharmacopoeia specifications for potency.

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## Colorectal cancer in patients under 50 years of age: A retrospective analysis of two institutions' experience

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### Abstract

**AIM:** To investigate the epidemiological characteristics of colorectal cancer (CRC) in patients under 50 years of age across two institutions.

**METHODS:** Records of patients under age 50 years of age who had CRC surgery over a 16 year period were assessed at two institutions. The following documents where reviewed: admission notes, operative notes, and discharge summaries. The main study variables included: age, presenting symptoms, family history, tumor location, operation, stage/differentiation of disease, and post operative complications. Stage of disease was classified according to the American Joint Committee on Cancer TNM staging system: tumor depth; node status; and metastases.

**RESULTS:** CRC was found in 180 patients under age 50 years (87 females, 93 males; mean age  $41.4 \pm 6.2$  years). Young patients accounted for 11.2% of cases during a 6 year period for which the full data set was

available. Eight percent had a 1<sup>st</sup> degree and 12% a 2<sup>nd</sup> degree family CRC history. Almost all patients (94%) were symptomatic at diagnosis; common symptoms included: bleeding (59%), obstruction (9%), and abdominal/rectal pain (35%). Evaluation was often delayed and bleeding frequently attributed to hemorrhoids. Advanced stage CRC (Stage 3 or 4) was noted in 53% of patients. Most tumors were distal to the splenic flexure (77%) and 39% involved the rectum. Most patients (95%) had segmental resections; 6 patients had subtotal/total colectomy. Poorly differentiated tumors were noted in 12% and mucinous lesions in 19% of patients of which most had Stage 3 or 4 disease. Twenty-two patients (13%) developed recurrence and/or progression of disease to date. Three patients (ages 42, 42 and 49 years) went on to develop metachronous primary colon cancers within 3 to 4 years of their initial resection.

**CONCLUSION:** CRC was common in young patients with no family history. Young patients with symptoms merit a timely evaluation to avoid presentation with late stage CRC.

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**Key words:** Colorectal cancer; Colorectal cancer screening; Sporadic colorectal cancer; Early-age onset colorectal cancer; Sigmoidoscopy

**Core tip:** Colorectal cancer (CRC) is rising among patients under age 50 years. In our study, the majority of patients did not have a family history of CRC and presented with advanced disease stages. In America, many physicians wrongly believe that CRC in patients under age 50 years is uncommon and mostly found in patients with a 1<sup>st</sup> degree family history of CRC. This misconception delays time to diagnosis, contributing to a more advanced disease stage on presentation. The authors hope, after reading this article, doctors will recommend timely and complete colon evaluations for

patients under age 50 years who present with rectal bleeding.

Myers EA, Feingold DL, Forde KA, Arnell T, Jang JH, Whelan RL. Colorectal cancer in patients under 50 years of age: A retrospective analysis of two institutions' experience. *World J Gastroenterol* 2013; 19(34): 5651-5657 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5651.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5651>

## INTRODUCTION

Colorectal cancer (CRC) remains a notable source of morbidity and mortality worldwide<sup>[1]</sup>. CRC is consistently the third most commonly diagnosed cancer in the United States. The American Cancer Society estimated that there would be 103170 new cases of colon and 40290 new cases of rectal cancer in 2012; in addition, 51690 deaths were predicted<sup>[2]</sup>. Although common, the overall incidence of CRC in the general population declined by 2.9% in men and 2.2% per year in women between 1998 and 2009<sup>[3]</sup>. An increase in the proportion of the population undergoing screening colonoscopy and the removal of benign precancerous polyps is thought to account for, at least, part of this decrease.

Patients with a first degree family history of CRC are advised to begin screening colonoscopy at age 40 or 10 years prior to the youngest age at which a family member with CRC was diagnosed. In addition, screening programs for CRC are now widely implemented for "average risk" patients, defined as those without a first degree family history of CRC or other risk factors, other than age. Universally, it is advised that screening begin at age 50 years for "average risk" patients. Asymptomatic patients under 50 years of age without a family history are excluded from almost all screening programs. Perhaps, in part, because of the age 50 years cut off many patients and doctors have a low index of suspicion for CRC in young patients without family history who present with bleeding or other symptoms. It is also the impression of many doctors and patients that the majority of young patients who develop CRC have a positive family history. Although less common than in older patients, sporadic CRC accounts for the majority of cases in patients under age 50 years.

The National Cancer Database Report on CRC noted that individuals under 50 years of age accounted for roughly 7% of all CRC in a 1990 study population of over 38000 patients<sup>[4]</sup>. As per the Surveillance Epidemiology and End Results (SEER) Program data from 1993 to 1997, patients younger than 55 accounted for roughly 12% of all CRC cases<sup>[5]</sup>. A recent study that examined data from the SEER Program cancer registries between 1992-2009 reported that the overall incidence of CRC per 100000 people (20-49 years age category) increased 1.6% and 1.7% per year in men and women, respectively,

over this time period<sup>[3]</sup>. A review of SEER Program data from 2005-2009 provides more detailed information regarding CRC in younger patients; in patients under 20 years the incidence was about 0.1%, in those 20-34 years of age it was 1.1%, in the 35-44 years sub-group the incidence was 4.0% whereas in the 45-54 years group it was 13.4%<sup>[2]</sup>. The data suggests that the incidence in younger patients is increasing<sup>[3]</sup>.

A number of reports regarding young patients with CRC have been published over the last few decades (Table 1); however, these reviews typically span many years, often include patients with familial syndromes and/or ulcerative colitis, and do not adequately comment on the relevant family history of the study patients<sup>[6-15]</sup>. This focused review regarding two institutions' experiences with patients under 50 years of age with CRC that came to surgery over a 16 year interval was conducted to determine: the volume of CRC operations for young patients, the proportion with a family history of CRC, the stage at presentation, the specific cancer location, and the presenting symptoms, if any.

## MATERIALS AND METHODS

### Patient population

Hospital records of patients under the age of 50 years who underwent CRC operations between July 1996 and May 2012 at two institutions were reviewed (New York Presbyterian Hospital, Columbia University Campus and St. Luke's Roosevelt Hospital, NY, United States). Specifically, the following documents were reviewed; admission notes, operative notes, discharge summaries, endoscopy records, and pathology reports. A subset of this data was also obtained from an IRB approved prospective data base of patients undergoing colorectal resection maintained by the senior author from 2006 to June 30, 2009 at New York Presbyterian Hospital and from July 1, 2009 until May 2012 at St. Luke's Roosevelt Hospital. This prospective database also provided the information regarding CRC patients over 50 years of age ( $n = 392$ ) used in this study. Additional information for this retrospective review was obtained from office charts and from telephone interviews.

### Study endpoints

The main study variables included: demographics, presenting symptoms leading to diagnosis, family history of CRC, tumor location, type of surgical resection, stage and differentiation of disease, and post operative complications. Patients with inflammatory bowel disease or known polyposis syndromes such as familial adenomatous polyposis syndrome, Gardner's syndrome and the like were excluded from the study.

Disease stage was reported according to the TNM Classification System used by the American Joint Committee on Cancer<sup>[16,17]</sup>. "T" refers to the size or direct extent of invasion of the primary tumor; "N" refers to the degree of spread to regional lymph nodes, if any; and "M"

**Table 1** Comparison of previously published reports of young patients with colorectal cancer

Ref.	Patients with CRC	Interval (yr)	Patient age (yr)	With family history of CRC (%) <sup>1</sup>
Recalde <i>et al</i> <sup>[6]</sup>	40	19	< 36	NR
Sanfelippo <i>et al</i> <sup>[7]</sup>	118	12	< 40	NR
Simstein <i>et al</i> <sup>[8]</sup>	41	15	< 40	4 (10)
Safford <i>et al</i> <sup>[9]</sup>	120 <sup>3</sup>	33	< 41	6 (5)
Pitluk <i>et al</i> <sup>[10]</sup>	31	10	< 40	1 (3)
Behbehani <i>et al</i> <sup>[11]</sup>	47 <sup>3</sup>	11	< 40	NR
Adloff <i>et al</i> <sup>[12]</sup>	32	7	< 40	NR
Domergue <i>et al</i> <sup>[13]</sup>	78 <sup>2</sup>	18	< 41	NR
Palmer <i>et al</i> <sup>[14]</sup>	105 <sup>2</sup>	12	< 40	NR
Fante <i>et al</i> <sup>[15]</sup>	90 <sup>2</sup>	9	< 51	18 (20)
Present series	180	16	< 50	20 (12) <sup>3</sup>

<sup>1</sup>Degree of relation not otherwise specified; <sup>2</sup>Series includes patients with familial adenomatous polyposis and/or ulcerative colitis; <sup>3</sup>Patients with first degree relatives with colorectal cancer. NR: Not reported; CRC: Colorectal cancer.

**Table 2** Patients' presenting signs and symptoms of colorectal cancer *n* (%)

Clinical presentation	Patients
Rectal bleeding	99 (57)
Anemia	19 (11)
Abdominal pain	54 (31)
Rectal pain	7 (4)
Change in bowel habits	37 (21)
Weight loss	20 (11)
Bowel obstruction	16 (9)
Perforation	5 (3)
Perforated diverticulitis	1 (0.6)
Screening	5 (3)
Unknown	7 (4)

refers to the presence of distant metastases.

### Statistical analysis

Statistical methods for comparing stage and tumor distribution between the under age 50 years and the 50 and over years groups included a 2-proportion Z test.

## RESULTS

### Patient demographics

One hundred eighty patients under 50 years of age (87 females, 93 males; range 17-49 years; mean 41.4 ± 6.2 years) underwent a CRC operation between July 1996 and May 2012 at the two institutions. In regards to the total number of patients (regardless of age) that underwent a CRC operation, complete data is only available for the period between July 2006 and May 2012; during this time period 437 CRC operations on adults were carried out of which 49 (11.2%) involved patients less than 50 years of age. When the total population of 180 patients under age 50 is considered, the distribution of CRC within age categories is as follows: under age 30 years, 8 patients (4%); age 30-39 years, 46 patients (26%); age 40-49 years, 126 (70%). Of note, 30% of the patients were younger than

**Table 3** Location of cancers *n* (%)

Anatomic location of cancer <sup>2</sup>	Cancers
Right colon <sup>1</sup>	31 (17.2)
Transverse colon	13 (7.2)
Descending colon	17 (9.4)
Descending and sigmoid colon junction	2 (1.1)
Sigmoid colon	29 (16.1)
Rectosigmoid colon	19 (10.6)
Rectum	71 (39.4)

<sup>1</sup>Right colon includes cecum, ascending, hepatic flexure; <sup>2</sup>Two patients had synchronous cancers.

40 years of age. One hundred and seventy patients (94%) reported symptoms upon presentation (Table 2).

### Family history of CRC

Family history data was available for 167 patients; 13 patients (7.8%) did not know their family history. Regarding the 167 patients with family history data, 14 patients (8.4%) had a first degree family history of CRC, 20 patients (12.0%) had a second degree history, and 6 patients (3.6%) had both a first and second degree family history of CRC. Thus, a total of 12% of patients had, at least, a first degree family history of CRC. Seventy six percent were sporadic CRC cases. One patient (age 42 years) presented with synchronous primary cancers of the cecum and splenic flexure and had a family history notable for 3 first and 1 second degree relatives with CRC. The Amsterdam criteria for hereditary non-polyposis colorectal cancer (HNPCC) were met in this patient<sup>[14]</sup>. Unfortunately, none of the patients in this series were evaluated for gene mutations associated with HNPCC (*i.e.*, *hMLH1*, *hMSH2*, *etc.*)<sup>[18]</sup>.

### Distribution of tumor location and colorectal resection

The majority of tumors (77%) were located distal to the splenic flexure, with 39% involving the rectum (Table 3). Proximal (right and transverse colon) cancers were noted in only 24% of patients. In comparison, a cohort of 392 CRC patients 50 years of age and older who underwent an operation between 2006 and 2012 at the same institutions by the same surgeons, presented with more proximal tumors (age < 50 years, 24% *vs* age ≥ 50 years, 43%; *P* < 0.0001) and fewer rectal tumors (age < 50 years, 39% *vs* age ≥ 50 years, 27%; *P* = 0.002) using 2-proportion Z-test (Table 4).

A formal colorectal resection was done in the majority of patients (95%), a transanal excision of a rectal cancer in 1 patient, colocolonic bypass for unresectable Stage 4 disease in 1 patient, and proximal diversion in 6 patients with obstructing, locally invasive cancers (Table 5). Twenty three abdominoperineal resections and 3 low anterior resections with mucosectomy and subsequent coloanal anastomosis were performed. A Hartmann's procedure was done in 4 patients with sigmoid or rectal lesions. Two patients (ages 34 and 42 years) had synchronous primary colon cancers and underwent subtotal colectomy. Twenty-

**Table 4** Colorectal cancer staging *n* (%)

Present series	Value	SEER <sup>2</sup>	Value
Age < 50 yr			
Stage 1 <sup>1</sup>	37 (21)	Localized	30%
Stage 2	47 (26)	Regional	40%
Stage 3	70 (39)	Distant	27%
Stage 4	26 (14)	Unstaged	3%
Age ≥ 50 yr			
Stage 1	88 (22)	Localized	38%
Stage 2	143 (36)	Regional	37%
Stage 3	135 (34)	Distant	19%
Stage 4	26 (7)	Unstaged	6%

<sup>1</sup>Two patients had Stage 0 disease and one patient had Tis disease following polypectomy; <sup>2</sup>Surveillance Epidemiology and End Results (SEER) 18, 2000-2009 stage distribution. Localized (confined to primary site), regional (spread to regional lymph nodes), distant (cancer has metastasized).

four distal resection patients were temporarily diverted (16% of all patients with anastomoses). Regarding the surgical methods used in the 172 patients who underwent bowel resection, the breakdown is as follows: laparoscopic-assisted, 78 patients (45.3%); hand-assisted or hybrid laparoscopic/open technique, 29 patients (16.9%); and open methods, 65 patients (37.8%).

### Staging distribution

According to the TNM system for cancer staging by the American Joint Committee on Cancer<sup>16,17</sup>, 37 patients (21%) had Stage 1 disease, 47 patients (26%) had Stage 2 disease, 70 patients (39%) had Stage 3 disease, and 26 patients (14%) had Stage 4 disease (Table 4). Three patients who underwent neoadjuvant chemoradiation for T3 rectal lesions based on preoperative endorectal ultrasound imaging had no residual disease on final pathology at the time of colorectal resection and were considered as Stage 2 lesions. Likewise, 3 patients who had sessile polyp cancers removed colonoscopically (invasion into submucosa noted on pathology) who underwent formal resection that revealed no residual cancer or involved lymph nodes on pathologic evaluation were classified as having Stage 1 cancers. Twenty patients with Stage 4 disease had known hepatic involvement and 3 patients had peritoneal carcinomatosis diagnosed at laparotomy.

Thirty-five patients (19%) underwent neoadjuvant chemoradiation, 3 patients (1.7%) underwent neoadjuvant chemotherapy alone, 10 patients (5.6%) underwent adjuvant chemoradiation, 56 patients (31%) underwent adjuvant chemotherapy, and 1 patient underwent adjuvant radiation alone for bony metastases. Of note, compared to the cohort of 392 CRC patients 50 years of age or older who underwent an operation between 2006 and 2012, patients under age 50 more often presented with Stages 3 and 4 disease (age < 50 years, 53% *vs* age ≥ 50 years, 41%; *P* = 0.003 using 2-proportion *Z*-test) (Table 4).

### Histopathological evaluation

Moderately or well differentiated cancers were noted in

**Table 5** Type of colon resection *n* (%)

Operation	Patients	Laparoscopic
Right colectomy	34 (19.8)	17 (50)
Transverse colectomy	2 (1.2)	2 (100)
Left colectomy	20 (11.6)	10 (50)
Descending and sigmoid colectomy	1 (0.6)	1 (100)
Sigmoid colectomy	22 (12.8)	15 (7)
Rectosigmoidectomy	24 (13.9)	21 (88)
Low anterior resection	40 (23.2)	26 (65)
Abdominoperineal resection	23 (13.4)	11 (48)
Subtotal/total colectomy	6 (3.5)	4 (67)
Total resections	172 (95.0)	107 (62)

124 patients (69%) whereas poorly differentiated cancers were found in 22 patients (12%). Of those with poorly differentiated histology, 67 percent presented with advanced Stage CRC (Stage 3 or 4). Thirty-four patients (19% of total) had mucinous adenocarcinomas of which 62% had advanced stage CRC.

### Postoperative complications and short-term outcomes

Regarding postoperative complications, there was 1 anastomotic leak and 4 intra abdominal/pelvic abscesses (reoperation in 1 patient, percutaneous drainage in 3 patients). Other postoperative complications included: ileus, 6 patients; small bowel obstruction, 6 patients (all required reoperation); wound infection, 7 patients; wound dehiscence, 2 patients (reoperation × 2); urinary retention, 3 patients; portal vein thrombosis, 1 patient; *C. difficile* colitis, 1 patient (treated with antibiotics); and incisional hernia, 1 patient (surgically repaired). There were no deaths perioperatively. Twenty-two patients (13%) developed recurrence and/or progression of disease to date. Three patients (ages 42, 42, and 49 years) went on to develop metachronous primary colon cancers within 3-4 years of their initial resection.

## DISCUSSION

It is well established that the incidence of CRC increases significantly beyond the 5<sup>th</sup> decade of life and continues to rise thereafter with increasing age. More recent reviews have shown that the percentage of CRC patients under 50 years of age has increased to approximately 12 percent<sup>15</sup>. Our data corroborates these findings as 11.2% of CRC patients in our study were younger than age 50 years.

Many people, lay and physician alike, falsely believe that the majority of patients under 50 years of age who develop CRC have a significant family history and are genetically predisposed to developing CRC. Interestingly, only 12% of patients in our study had a first degree relative with CRC and only 1 patient (age 42 years) met the Amsterdam criteria for HNPCC based on family history. A literature search revealed several reports regarding young patients with CRC that showed family history data<sup>16-15</sup> similar to that of our study findings. In the general population of CRC patients (all ages), an estimated 15%-20% of patients have a family history of colorectal

neoplasia<sup>[19]</sup>. Therefore, regardless of age at diagnosis, the vast majority of patients with CRC have sporadic disease and are “average risk” patients without a family or personal history of colorectal neoplasm, inflammatory bowel disease, polyposis syndromes, or other risk factors.

Are CRCs in the under 50 population different from tumors that occur in older patients? In the absence of detailed genetic analyses of the tumors we must use clinical and basic pathologic data to address this question. The stage breakdown data may be helpful in this regard, although it is influenced by factors other than the tumors’ biologic aggressiveness (*i.e.*, the timeliness of diagnosis). Similarly, the differentiation profile of the tumors in the younger and older CRC populations permits comparison of the 2 groups.

SEER stage distribution data from 2000-2009 for individuals with CRC under 50 years of age noted that 30% had localized disease (confined to primary site), 40% had regional disease (spread to mesenteric lymph nodes), and 27% had distant disease (metastatic) at the time of diagnosis; thus, 67% had Stage 3 or 4 disease. In contrast, in the over 50 age group, 39% of patients had localized disease, 37% had regional disease and 19% had distant disease at diagnosis; thus, 56% had Stage 3 or 4 disease<sup>[2]</sup>. Our study results, as well as other investigators<sup>[8,10,11,13]</sup>, support the notion that advanced Stage (Stage 3 or 4) at presentation is more common among young patients compared to older patients (50 years and older). Taken together, the available data suggests that younger patients with CRC more often present with advanced disease when compared to the general population<sup>[8,10,11,13]</sup>.

Regarding tumor differentiation, previous studies report a greater percentage of poorly differentiated (19%-49% of total) and mucinous tumors (9%-49%) in younger CRC populations<sup>[3,8,20,21]</sup>, whereas, in the current study, only 12% of patients had poorly differentiated adenocarcinomas and 20% had mucinous histology. Data concerning the general CRC population from two previously published studies suggests that about 15% of colorectal adenocarcinomas are poorly differentiated and 17% demonstrate mucinous histology<sup>[22,23]</sup>. The relatively low percentage of poor prognosis histologies in our study may be related, in part, to the small number of HNPCC patients in the study population since HNPCC is associated with a higher incidence of poorly differentiated and mucin producing tumors. Regardless, the histology data does not provide an explanation for the high incidence of advanced stage tumors seen in the current study population. As mentioned earlier, there may be factors other than unfavorable histology and aggressive tumor biology contributing to the high rate of advanced stage tumors seen in younger CRC patients.

It has been suggested that delays in diagnosis may account, in part, for the advanced Stage at presentation noted in many CRC patients under 50 years of age<sup>[14,24]</sup>. Young symptomatic patients may delay presentation to a physician out of ignorance, fear, or denial. Furthermore, when confronted with young, average risk patients, clini-

cians may attribute their symptoms to any number of common benign anorectal disorders. The already mentioned fixation on 50 years as the age after which CRC occurs likely figures into the practitioner’s thinking as well. Consequently, a full colorectal evaluation may not be carried out for months to years following the onset of symptoms. For example, in the current study multiple patients reported a history of intermittent rectal bleeding and were treated for “hemorrhoids” for a year or longer before referral for diagnostic endoscopy. In one patient with an 18 mo history of diarrhea and occasional bleeding with mucus, a corresponding note from a gastroenterologist stated that the change in bowel habits likely represented irritable bowel syndrome with hemorrhoids. This patient ultimately underwent a colonoscopy and was found to have a sigmoid cancer. Another patient who reported postpartum rectal bleeding was told by her family practitioner that the cause was most likely hemorrhoids. This patient was eventually referred for a colonoscopy two years later after developing abdominal pain at which time she had a palpable rectal mass; she proved to have a Stage 3 lesion.

The ramifications of delayed diagnosis, specifically presentation with a more advanced CRC stage with its attendant increased mortality, justify, in the authors’ opinion, prompt and complete large bowel evaluation in all patients under 50 years of age who present with suspicious colorectal symptoms in order to “rule out” an occult neoplasm. Signs and symptoms that may prompt such an evaluation include: bleeding, heme-positive stool, anemia, changes in stool caliber or bowel habits, and abdominal pain or persistent distension of unclear etiology.

### **Current screening and surveillance guidelines**

There are currently no screening guidelines in place by the American Society of Colon and Rectal Surgeons (ASCRS) or the American Cancer Society (ACS) pertaining specifically to young patients who present with symptoms that could signify an underlying neoplasm. The ASCRS<sup>[25]</sup> does recommend, in general, that anyone “with symptoms or signs that suggest the presence of CRC or polyps who fall outside the domain of screening should be offered an appropriate diagnostic evaluation.” However, it appears that in the United States too few practitioners are following this recommendation when confronted with young patients who report bleeding or other symptoms.

Questions regarding cancer surveillance and screening for patients and their families commonly arise when caring for young CRC patients. The following guidelines from the ASCRS and the ACS have evolved over the last 3 decades; although there are some minor differences they are quite similar. People with a first-degree relative (parent, sibling, or child) who has had CRC or adenomatous polyp(s) are advised to have screening colonoscopy starting at age 40 or ten years younger than the age, at diagnosis, of the youngest family member with CRC (whichever comes first) with repeated evaluation every

5 years. Individuals with two second degree relatives (grandparent, aunt, or uncle) with CRC or polyps are advised to be screened as average risk patients (see below), but beginning at age 40 years. Individuals with a single second or third degree relative (cousin, great-grandparent) with CRC or polyp(s) are advised to follow average risk screening guidelines. Individuals in HNPCC kindreds are advised to begin colonoscopic evaluation starting at 20-25 years of age or 10 years before the age of diagnosis in the youngest CRC patient in the immediate family, whichever comes first<sup>[25]</sup>.

In average risk patients the ASCRS<sup>[25]</sup> recommends that routine screening commence at age 50 years. As per the ASCRS, acceptable screening strategies for average risk patients include: (1) flexible sigmoidoscopy every 5 years; (2) a double contrast barium enema every 5-10 years; or (3) a colonoscopy every 10 years. The ACS recommendations are almost identical to those of the ASCRS except that they include virtual colonoscopy every 5 years as an acceptable screening option. In regards to average risk patients under age 50 years without family history, the ASCRS<sup>[25]</sup> advises that they commence annual digital rectal examination and fecal occult blood testing at age 40 years; the ACS makes no recommendations for this group.

### Future directions

Is endoscopic screening indicated for asymptomatic patients under 50 years of age? The rising incidence of CRC in this group and the tendency towards advanced Stage at presentation would argue in favor of such programs. Yet, the cost of screening colonoscopy programs would be very high and given the current economic climate and the fact that the incidence of CRC is still considerably lower in this group (*vs* the over age 50 population) the initiation of such programs is highly unlikely. However, perhaps a case can be made for a single screening sigmoidoscopy at age 40. In the present series a screening sigmoidoscopy would most likely have revealed a significant number of the neoplasms. As per the ACS, sigmoidoscopy identifies 70%-80% of individuals with advanced lesions and is associated with a 60%-80% reduction in CRC mortality for the area of the colon within its reach. Furthermore, in a recent multi-center randomized trial a single screening sigmoidoscopy carried out between the ages of 55 and 64 reduced CRC incidence by 33% and mortality by 43%<sup>[26]</sup>. Sigmoidoscopy, although invasive, is a safe procedure with a very low rate of perforation that is well tolerated without sedation. Although the chances for successful initiation of a flexible sigmoidoscopy screening program for young patients are small, the authors believe that our current dismal record with young CRC patients justifies the effort.

### Study limitations

The lack of genetic testing for HNPCC in this study population is a clear weakness of this study. Similarly, the lack of long term cancer recurrence and survival results is

also a major shortcoming. Ideally, genetic categorization of the tumors *via* microarray would be done which would permit a detailed analysis and comparisons.

In conclusion, this review corroborates recent national data regarding the rising incidence of CRC in the under age 50 population and the fact that a greater percentage of younger patients present with Stages 3 and 4 disease when compared to the entire CRC population. The vast majority of the young patients in this study had sporadic CRC; only 12% had a first degree family history of CRC and only 1 patient met the clinical criteria for HNPCC. The histologic breakdown of the tumors was similar to that reported in the general population of CRC patients.

Although it is impossible to confirm, it is the impression of the authors that in many patients there was a delay, either by the patient, physician, or both, in carrying out the appropriate diagnostic evaluation. The widely held belief that CRC occurs in patients age 50 and older likely contributes to this mind set. Clearly, at the very least, young symptomatic patients with rectal bleeding, a change in bowel habits, or abdominal pain should be promptly evaluated, preferably with a full colonoscopy. Finally, the medical and surgical community need to consider the concept of some type of screening program for young patients, perhaps flexible sigmoidoscopy, beginning at age 40 years. The goal is to diagnose CRC in this population at an earlier stage so that the recurrence rates and mortality can be reduced. The medical community and the public must be made aware of the fact that CRC occurs in patients under age 50 years with regularity and that thorough evaluation is indicated for symptomatic patients regardless of their age.

## COMMENTS

### Background

While the incidence of colorectal cancer (CRC) is declining in the overall population, it is on the rise among individuals under 50 years of age. Many patients and practitioners, alike, believe most cases of early-age onset CRC are attributed to a family history of CRC; however, a growing number of small studies now show that the majority of these young patients have no family history.

### Research frontiers

A growing number of small studies are being published that examine the rising incidence of CRC among patients under age 50 years. It is becoming increasingly evident that the majority of these patients do not have a family history of CRC; however, it is unclear what predisposition these patients have to develop CRC at an earlier age. The research hotspot may be to perform genetic categorization of these tumors *via* microarray analysis to determine how they differ from sporadic CRC in older patients.

### Innovations and breakthroughs

Previous studies in the literature that have examined the incidence of early-age onset CRC often include patients with hereditary, familial, and sporadic CRCs (see below). In the study, the authors excluded patients with a known genetic CRC predisposition or history of inflammatory bowel disease. The vast majority of patients under age 50 years in the study had no reported family history of CRC and did not meet criteria by family history for hereditary nonpolyposis colorectal cancer (HNPCC). Therefore, the authors believe the majority of patients in the study had early-age onset "sporadic" CRC similar to the general population.

### Applications

The study corroborates the findings of several other studies evaluating early-age onset CRC in that the majority of patients have no family history of CRC and most tumors were found in the distal colon and rectum. This contradicts the

believe of many practitioners that early-age onset CRC's are more often located in the proximal colon and are attributable to inherited predispositions (*i.e.*, hereditary non-polyposis colorectal cancer). The next logical step is to investigate what triggers the development of a "sporadic" CRC at a younger age of onset among some individuals.

### Terminology

Familial CRC is defined as CRC that presents 10-20 years earlier than the general population with no clear inheritance pattern. It is presumed that lower-penetrance susceptibility genes may play a role. Hereditary CRC accounts for approximately 5%-10% of patients diagnosed with CRC. Examples include familial adenomatous polyposis syndrome and HNPCC (Lynch Syndrome), which show autosomal dominant inherited germline mutations. Tumors are often located in the proximal colon and can be synchronous or metachronous in nature. Sporadic CRC occurs in patients without identifiable genetic predispositions or a reported family history of CRC.

### Peer review

This is a descriptive study in which the authors investigate the incidence of early-age onset CRC among two institutions. The results corroborate a growing body of literature that now suggests that most CRC's, regardless of age of onset, occur without a particular genetic or familial predisposition.

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## Cardiovascular disease risk factor profiles in children with celiac disease on gluten-free diets

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### Abstract

**AIM:** To describe the cardiovascular disease (CVD) risk factors in a population of children with celiac disease (CD) on a gluten-free diet (GFD).

**METHODS:** This cross-sectional multicenter study was performed at Schneider Children's Medical Center of Israel (Petach Tikva, Israel), and San Paolo Hospital (Milan, Italy). We enrolled 114 CD children in serologic remission, who were on a GFD for at least one year. At enrollment, anthropometric measurements, blood lipids and glucose were assessed, and compared to values at diagnosis. The homeostasis model assessment-estimated insulin resistance was calculated as a measure of insulin resistance.

**RESULTS:** Three or more concomitant CVD risk factors [body mass index, waist circumference, low density lipoprotein (LDL) cholesterol, triglycerides, blood pressure and insulin resistance] were identified in 14% of CD subjects on a GFD. The most common CVD risk factors were high fasting triglycerides (34.8%), elevated blood pressure (29.4%), and high concentrations of calculated LDL cholesterol (24.1%). On a GFD, four children (3.5%) had insulin resistance. Fasting insulin and HOMA-IR were significantly higher in the Italian cohort compared to the Israeli cohort ( $P < 0.001$ ). Children on a GFD had an increased prevalence of borderline LDL cholesterol (24%) when compared to values (10%) at diagnosis ( $P = 0.090$ ). Trends towards increases in overweight (from 8.8% to 11.5%) and obesity (from 5.3% to 8.8%) were seen on a GFD.

**CONCLUSION:** This report of insulin resistance and CVD risk factors in celiac children highlights the importance of CVD screening, and the need for dietary counseling targeting CVD prevention.

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**Key words:** Celiac disease; Cardiovascular disease risk factors; Gluten-free diet; Insulin resistance; Children; Hyperlipidemia; Cholesterol

**Core tip:** In our study we demonstrate a relatively high proportion of children with celiac disease (CD) adherent to a gluten-free diet (GFD) with one or more cardiovascular disease (CVD) risk factors. Furthermore, this is the first report of insulin resistance in celiac patients either in adults or children. These findings suggest that screening for CVD risk factors in celiac children both at diagnosis and during follow-up is important. Furthermore, dietary counseling over time, targeting obesity and CVD risk factors in addition to monitoring adherence to a GFD in children and adolescents diagnosed with CD, may be warranted.

Norsa L, Shamir R, Zevit N, Verduci E, Hartman C, Ghisleni D, Riva E, Giovannini M. Cardiovascular disease risk factor profiles in children with celiac disease on gluten-free diets. *World J Gastroenterol* 2013; 19(34): 5658-5664 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5658.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5658>

## INTRODUCTION

Celiac disease (CD) is a common gastrointestinal autoimmune disorder characterized by inflammation of the small bowel mucosa triggered and sustained by ingestion of gluten in genetically predisposed individuals<sup>[1]</sup>. The prevalence of CD worldwide ranges between 0.5% and 3% of the general population<sup>[2-4]</sup>. Although CD has traditionally been considered a malabsorptive disorder associated with diarrhea and weight loss, these symptoms are now seen less frequently<sup>[5]</sup>. Several recent studies have reported that only a minority of newly diagnosed patients were underweight. Instead, many patients, both children and adults were overweight or even obese<sup>[6,7]</sup>.

A definitive diagnosis of CD is currently made using IgA anti-tissue transglutaminase (tTG) antibody screening followed, in most cases, by confirmatory biopsies of the small intestine with compatible histopathological findings<sup>[8]</sup>. Currently, the only treatment for CD is a strict, life-long gluten-free diet (GFD) which leads to rapid clinical improvement, especially in children. The normalization of serological tests usually occurs 6 to 12 mo after initiation of a GFD<sup>[2]</sup>.

Deranged adiposity, blood lipid profile abnormalities and other risk factors for cardiovascular disease (CVD) in CD patients are still debated, and clear conclusions have yet to be reached<sup>[9]</sup>. Several studies have demonstrated that CVD risk factors, namely obesity, abnormal blood lipid profile, hypertension and insulin resistance have their roots in childhood and tend to track into adulthood<sup>[10-12]</sup>. The primary aim of this study was to describe CVD risk factors in a population of celiac children adherent to a GFD for at least one year, in two Mediterranean countries.

## MATERIALS AND METHODS

This cross-sectional multicenter study was performed at Schneider Children's Medical Center of Israel (Petach Tiqva, Israel), and San Paolo Hospital (Milan, Italy) between June 2010 and December 2011.

The study population included individuals less than 18 years old, previously diagnosed with CD, without known co-morbidities, who were referred for follow-up to the pediatric gastroenterology clinics of the participating centers. Patients were included if they had a definitive diagnosis of CD, ascertained by both positive serology and confirmed by compatible duodenal biopsies, and who had been on a GFD for at least one year with complete normalization of their CD serology (tTG antibodies) at

the time of enrollment.

At enrollment, all patients underwent physical examination including measurement of weight using a digital scale, and height using a stadiometer. Measurement of waist circumference was performed with a tape measured to the nearest 0.5 cm at the midpoint between the bottom of the rib cage and the top of the iliac crest. Blood pressure was measured in the sitting position using an appropriately sized cuff. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. To evaluate BMI values across different age and gender groups we used the BMI standard deviation score percentiles that were calculated according to the Center of Disease Control and Prevention growth charts of 2000<sup>[13]</sup>. Children with BMI values lower than the 5<sup>th</sup> percentile for age and gender were classified as "underweight", those in the 5<sup>th</sup> to 85<sup>th</sup> percentile were classified as "normal weight", those in the 85<sup>th</sup> and 95<sup>th</sup> percentile were classified as "overweight" and those greater than the 95<sup>th</sup> percentile were classified as "obese"<sup>[13]</sup>. Pre-hypertension was defined as an average systolic or diastolic blood pressure between the 90<sup>th</sup> and 95<sup>th</sup> percentile for sex, age, and height-percentile-specific, and hypertension if the values were above the 95<sup>th</sup> percentile<sup>[14]</sup>. The same pediatrician performed the Tanner stage of puberty.

After an overnight fast of at least 8 h, blood samples were drawn for fasting glucose and insulin, triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol and tTG antibodies. The samples were analyzed in local laboratories. tTG antibody levels were quantified by enzyme linked immunosorbent assay. Serum glucose level was measured by the enzymatic UV test method using an automated analyzer and total cholesterol, triglycerides, and HDL cholesterol concentrations were measured by an enzymatic colorimetric method on an automated analyzer. LDL cholesterol was calculated using the Friedewald equation: LDL cholesterol = total cholesterol - [HDL cholesterol + (triglyceride/5)]. Serum insulin concentrations were measured by an immunometric assay with the Immulite 2000 Analyzer. According to the American Academy of Pediatrics (AAP) criteria, borderline levels of cholesterol were defined by values between the 75<sup>th</sup> and 95<sup>th</sup> percentile of LDL cholesterol, while values greater than the 95<sup>th</sup> percentile were considered elevated<sup>[15]</sup>. Insulin resistance was estimated by the homeostatic model assessment (HOMA-IR), as follows: HOMA-IR = [fasting insulin ( $\mu$ U/mL)  $\times$  fasting glucose (mmol/L)/22.5]. Although the hyperinsulinemic euglycemic clamp is the only validated method to evaluate insulin sensitivity in the pediatric population, HOMA has been widely used to estimate insulin resistance in the screening of large populations of euglycemic children<sup>[16]</sup>. In this study, insulin resistance was defined as HOMA > 3.16 according to the most recent cut-off for the pediatric population<sup>[17]</sup>. Based on the Bogalosa Heart Study<sup>[11]</sup>, we analyzed six risk factors for CVD. The CVD risk factors considered were BMI Z-scores greater than the 85<sup>th</sup> percentile, waist circumference over the 90<sup>th</sup> percentile<sup>[18]</sup>, fasting LDL cholesterol or triglycerides higher than the 75<sup>th</sup> percentile,

**Table 1** Descriptive data of the study populations at diagnosis (T0) and recruitment (T1)

Variable	Israel	Italy	P-value
<i>n</i>	70	44	
Sex (female)	71.40%	59.10%	0.176
Age at diagnosis (mo)	77.0 ± 43.5	68.7 ± 48.5	0.185
Duration of GFD (mo)	38.9 ± 30.4	69.7 ± 55.6	< 0.0001 <sup>1</sup>
Weight Z-score			
T0	-0.405 ± 1.21	-0.931 ± 1.36	0.357
T1	-0.172 ± 1.25	-0.240 ± 1.19	0.881
Height Z-score			
T0	-0.397 ± 1.14	-0.682 ± 1.40	0.455
T1	-0.192 ± 1.78	-0.310 ± 1.02	0.227
BMI Z-score			
T0	-0.103 ± 1.1	-0.369 ± 1.0	0.489
T1	-0.025 ± 1.2	-0.162 ± 1.2	0.760

<sup>1</sup>Statistically significant. Values are mean ± SD or number of subjects (percentage). BMI: Body mass index; GFD: gluten-free diet.

systolic or diastolic blood pressure greater than the 90<sup>th</sup> percentile and the state of insulin resistance<sup>[19]</sup>.

In addition, we retrieved all the available data on anthropometry, blood lipids and glucose profiles at the time of diagnosis of CD from patient files.

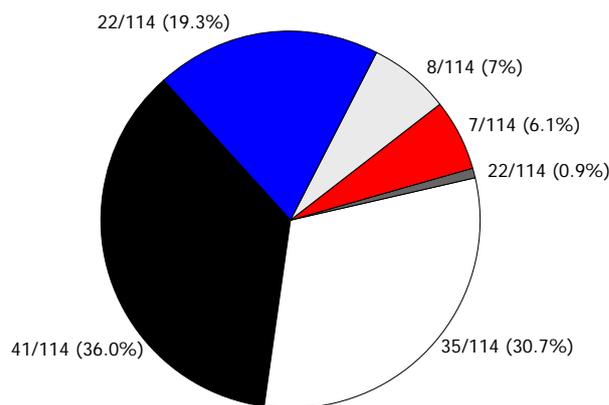
This study was approved by the institutional review boards at each of the participating centers. Signed informed consent was provided by a legal guardian of each participant.

### Statistical analysis

Descriptive data are shown as mean ± SD or number of observations (percentage). Symmetry of distribution of the variables was tested by the Kolmogorov-Smirnov test (*P* > 0.05). Triglycerides were not symmetrically distributed and were therefore log-transformed for analysis. Comparisons between the groups for continuous variables were performed by the *t* test for unpaired data or the Wilcoxon-Mann-Whitney test as appropriate. The  $\chi^2$  test for unpaired discrete variables and the Wilcoxon-Mann-Whitney test for paired discrete variables were used in this study. Additionally, a multiple logistic regression analysis was performed to assess the independent association of the center with HOMA-IR, adjusted for age, gender, BMI Z-score, Tanner stage and duration of diet. All *P*-values less than 0.05 were considered to indicate statistical significance (two tailed test). The SPSS software, version 18.0 (SPSS Inc, Chicago, IL, United States) was used for the statistical analysis.

## RESULTS

During the study period, 114 children with a mean age of 10.4 (± 4.1) years (70 from Israel and 44 from Italy) were enrolled. The two populations of children had comparable demographic data and anthropometry both at diagnosis and after at least 1 year of a GFD (Table 1). The only significant difference was a longer follow-up period in the Italian children. Complete fasting lipid profiles prior to



**Figure 1** Risk factors for cardiovascular disease in pediatric patients with celiac disease in serological remission on gluten-free diets. White: 0 risk factor; Black: 1 risk factor; Blue: 2 risk factors; Light gray: 3 risk factors; Red: 4 risk factors; Dark gray: 5-6 risk factors. Risk factors sought included BMI Z-scores greater than the 85<sup>th</sup> percentile, waist circumference over the 90<sup>th</sup> percentile<sup>[18]</sup>, fasting low density lipoprotein cholesterol or triglycerides higher than the 75<sup>th</sup> percentile, blood pressure systolic or diastolic greater than the 90<sup>th</sup> percentile and insulin resistance<sup>[19]</sup>.

initiation of the GFD were available for 52/114 children, 36/70 from Israel and 16/44 from Italy, and insulin levels were not available from CD diagnosis as prior to our study the screening of lipid and glucose profiles was not routinely performed in patients with suspected CD.

### CVD risk factors

Overall, 14% of the cohort had 3 or more concomitant risk factors (Figure 1). Only 30.7% of the cohort did not have any risk factors (Figure 1). No significant difference was seen in the prevalence of CVD risk factors between the two countries in the cohort. The most common CVD risk factors were high fasting triglycerides (34.8%), elevated blood pressure (29.4%), and high concentrations of calculated LDL cholesterol (24.1%).

### Anthropometry

We did not find any significant difference in the anthropometrics data between the Israeli and Italian CD children (Table 1). Anthropometrics in the whole cohort prior to and following the introduction of a GFD revealed significant increases in both height and weight Z-scores with an increase in BMI Z-scores which did not reach significance (Table 2). When scores were pooled into the CDC BMI categories, we found that both the prevalence of overweight and obesity increased from 8.8% and 5.3%, respectively, at the time of diagnosis to 11.4% and 8.8%, respectively, after the introduction of a GFD. This trend did not attain statistical significance (*P* = 0.105).

### Lipid profile

There were no significant differences in the lipid profiles between the Israeli and Italian cohorts, except for higher levels of HDL cholesterol in the Italian patients. According to AAP criteria, 63% of the patients in our cohort had normal LDL cholesterol, 30% had borderline and

**Table 2** Changes in height, weight, and body mass index at diagnosis (T0) and recruitment (T1) *n* (%)

Variable	T0	T1	P-value
Z-score height	-0.447 ± 1.1	-0.238 ± 1.1	0.001 <sup>1</sup>
Z-score weight	-0.567 ± 1.3	-0.198 ± 1.2	< 0.001 <sup>1</sup>
BMI Z-score	-0.207 ± 1.1	-0.078 ± 1.2	0.103
BMI categories <sup>2</sup>			
Underweight	11/114 (9.6)	12/114 (10.5)	
Normal weight	87/114 (76.3)	79/114 (69.3)	
Overweight	10/114 (8.8)	13/114 (11.4)	
Obese	6/114 (5.3)	10/114 (8.8)	0.105

<sup>1</sup>Statistically significant; <sup>2</sup>Classification<sup>[13]</sup>. Values are mean ± SD or number of subjects (percentage). BMI: Body mass index.

7% had hypercholesterolemia after at least one year of a GFD (Table 2). Although data on the lipid profile before CD diagnosis were available only in 50% of enrolled patients, we found significant increases in both total cholesterol and HDL cholesterol in patients on a GFD. The categorization of the LDL cholesterol values highlighted an increase in the prevalence of borderline cholesterol levels (from 9.6% to 23.1%), which did not reach statistical significance ( $P = 0.090$ ).

### Insulin resistance

The Italian children were found to have both higher fasting insulin and HOMA-IR levels while on a GFD when compared to the Israeli cohort (Table 3). Four patients (3.5%) were identified with frank insulin resistance, three from Italy, and one from Israel (Table 3). Two of these had normal weight and the remaining patients were overweight.

## DISCUSSION

This cross-sectional study is the first to describe the profile of CVD risk factors in a cohort of children with CD in serologic remission on a GFD. Furthermore, this is the first report of insulin resistance in children with CD on a GFD.

Less than one third of our cohort did not have any CVD risk factors, while 14% had three or more risk factors. This finding suggests that CVD screening may be important in pediatric CD patients both at diagnosis and during follow-up. Studies have demonstrated that an earlier onset and greater number of CVD risk factors increase the chance of atheromatous plaque formation<sup>[10,11]</sup>.

Our study design, which did not include a healthy control group, did not intend to determine whether children with CD have a higher risk than the general population for the development of CVD. Further prospective studies are needed to evaluate if changes in lifestyle and environment are responsible for a higher cardiovascular risk in celiac patients compared with the normal population. Nevertheless, although this study is limited by the lack of data prior to initiation of a GFD, it may suggest that the clinical and dietary follow-up should target adiposity, lipid profile and other CVD risk factors in addition to the common practice of dietary monitoring of

**Table 3** Lipidic, glycemic and insulinemic profile at enrollment in the two populations *n* (%)

Variable	Israel	Italy	P-value
Total cholesterol (mg/dL)	158.3 ± 27.6	162.5 ± 24.9	0.570
Cholesterol LDL (mg/dL)	95.4 ± 21	89.9 ± 22.3	0.116
Cholesterol HDL (mg/dL)	49.9 ± 10.6	59.4 ± 12.4	< 0.001 <sup>1,3</sup>
Triglycerides (mg/dL)	71.1 ± 25.2	62.7 ± 20.9	0.055
LDL cholesterol classes <sup>2</sup>			
Normal	44/70 (62.9)	28/44 (63.6)	
Borderline	22/70 (31.4)	12/44 (27.3)	
Hypercholesterolemia	4/70 (5.7)	4/44 (9.1)	0.742
Glucose (mg/dL)	83.4 ± 6.9	80.3 ± 8.8	0.046 <sup>1,3</sup>
Insulin $\mu$ U/mL	3.3 ± 2.7	7.5 ± 4.3	< 0.001 <sup>1,3</sup>
HOMA index	0.69 ± 0.6	1.55 ± 1.0	0.001 <sup>1,3</sup>
Insulin resistance			
HOMA-IR < 3.16	69/70 (98.6)	41/44 (93.2)	
HOMA-IR > 3.16	1/70 (1.4)	3/44 (6.8)	0.108

<sup>1</sup>Statistically significant; <sup>2</sup>AAP classification<sup>[15]</sup>; <sup>3</sup>P-values are adjusted for age, gender, body mass index (BMI) Z-score, Tanner stage and duration of diet. Values are mean ± SD or number of subjects (percentage). HOMA-IR: Homeostasis model assessment-estimated insulin resistance; LDL: Low density lipoprotein.

adherence to a GFD.

The introduction of a GFD in CD patients increases the intestinal absorption of both macro and micro-nutrients. This leads to improved weight and height in celiac children presenting with malabsorption (weight loss, failure to thrive, poor weight gain)<sup>[20]</sup>. In our cohort, the majority of patients were of normal weight at the time of diagnosis and the percentage of overweight or obese patients was higher than those who were underweight. This drift in clinical presentation is concordant with previous reports<sup>[7]</sup> and may be attributed to increased awareness and early diagnosis. Alternatively, it may be explained by the radical change in diet and lifestyle in developed countries in recent decades, in line with the increasing prevalence of overweight and obesity in the general population. The increased prevalence of overweight and obesity after the introduction of a GFD in this study, although not significant ( $P = 0.10$ ), may suggest the potential of a GFD to increase weight even in children presenting as normal or overweight at the time of CD diagnosis. The influence of a GFD on BMI remains unclear both in adults and children<sup>[9]</sup>. In adults, the debate is mainly based on two discordant theories. Dickey *et al.*<sup>[21]</sup> demonstrated further weight gain in patients already overweight at the time of CD diagnosis after the introduction of a GFD, while Cheng *et al.*<sup>[22]</sup> showed a positive effect of a GFD by demonstrating weight gain in previously underweight patients and weight loss in those previously overweight. Furthermore a recent study<sup>[23]</sup> recruiting a very large cohort of adult patients found that strict GFD adherence could increase the prevalence of overweight and obesity in CD patients. Contrasting studies have also recently appeared in the pediatric literature. Valletta *et al.*<sup>[24]</sup> reported an increase in the fraction of overweight children following the introduction of a GFD, while Brambilla *et al.*<sup>[25]</sup> demonstrated a beneficial effect of GFD on BMI in the

majority of CD children. Reilly *et al.*<sup>[26]</sup> found a beneficial effect of GFD on the BMI of overweight celiac children. Our data, demonstrating that a GFD increases the prevalence of overweight and obesity in children with CD, is in agreement with studies reporting increased weight as a potential adverse effect of GFD.

The data concerning LDL cholesterol after at least one year of a GFD suggests an important role for cholesterol as a CVD risk factor in our cohort. In this study, LDL cholesterol was the third most prevalent CVD risk factor in celiac children on a GFD.

The increase in total and HDL cholesterol after GFD introduction in comparison to levels prior to initiation of a GFD (available from a subset of patients), is concordant with some studies which theorized that derangement of intestinal absorption, chylomicron production and lipoprotein metabolism may underlie the finding of lower levels of total and HDL cholesterol in untreated CD, which can revert to normal after treatment<sup>[27-30]</sup>. In contrast, we found that the rate of borderline LDL cholesterol concentrations more than doubled (from 9.6% to 23.1%) following adherence to a GFD. This may be the result of a tendency in adult and adolescent patients to consume gluten-free products with high fat contents<sup>[31-33]</sup> in order to compensate for the withdrawal of common gluten-containing carbohydrates from the diet.

Our data seem to suggest that although the increase in the rate of borderline LDL cholesterol could raise the cardiovascular risk, the concomitant increase in HDL may be cardioprotective, and thus future studies looking at surrogate markers of atherosclerosis are needed to determine whether a GFD is harmful in this regard.

Four children (3.5%) on a GFD had insulin resistance. As far as we are aware, the only studies reporting HOMA-IR in CD were performed in patients with concomitant insulin-dependent diabetes mellitus (IDDM) 1<sup>[34]</sup>. It is not known whether insulin resistance was present on CD diagnosis. As such, this is the first description of the presence of insulin resistance in CD children.

Due to the lack of insulin levels before CD diagnosis, we were unable to assess whether such insulin resistance is directly related to the introduction of a GFD. Previous publications have reported that available gluten-free products (*e.g.*, gluten-free bread, pasta, pizza *etc.*) have much higher glycemic indices than their gluten-containing equivalents, ingestion of which may lead to increased secretion of insulin<sup>[35-37]</sup>. Our findings, along with the previously mentioned change in the pattern of CD presentation, may suggest that future assessment of fasting glucose and insulin in children diagnosed with CD before and during the introduction of GFD should be performed. This is especially true in light of the role of insulin resistance as a CVD risk factor, and a predisposing condition for the development of type 2 diabetes<sup>[19]</sup>. The significantly higher fasting insulin levels and HOMA-IR in our Italian cohort may be explained by genetic and dietary differences between the two groups<sup>[37]</sup>. Our findings suggest that despite the classical consideration of CD as a malabsorptive condition, metabolic derangements, gen-

erally not attributed to this condition, should be actively sought even in patients who are non-obese. Although our data may hint to insulin resistance as a new complication of CD, a word of caution is due, as this study was performed on a cohort of CD patients and data is lacking in the literature regarding the prevalence of glucose intolerance in the healthy, non-overweight/obese children and adolescents.

This study has a number of limitations such as the relatively small number of patients, the cross-sectional design which did not allow for pre-GFD levels of all measured parameters, and the lack of familial history for CVD risk factors which may have further impacted our findings. However, despite these limitations, we have described the presence of insulin resistance in pediatric CD for the first time, and specifically addressed other CVD risk factors in the pediatric CD population on a GFD in serological remission.

Prior to initiation of the study, the relationship between CD and CVD risk factors was not clear, and therefore screening of lipid and glucose profiles was not routinely performed in patients with suspected CD. Additionally, the similarity in most findings between patients from two different countries, suggest that these findings are neither geographically nor ethnically specific. Prospective studies are needed to delineate the role of the GFD in the development of CVD risk factors in celiac children.

In conclusion, this cross-sectional study demonstrates a relatively high proportion of children with CD adherent to a GFD with one or more CVD risk factors including insulin resistance. These findings suggest the importance of screening for CVD risk factors in celiac children both at diagnosis and during follow-up. Furthermore, dietary counseling over time, targeting obesity and CVD risk factors in addition to monitoring adherence to a GFD in children and adolescents diagnosed with CD, may be warranted.

## COMMENTS

### Background

Celiac disease has traditionally been considered a malabsorptive disorder associated with diarrhea and weight loss, however, these symptoms are now seen less frequently. Several recent studies have reported that only a minority of newly diagnosed patients were underweight. Instead, many patients, both children and adults were overweight or even obese.

### Research frontiers

Several studies have demonstrated that cardiovascular disease (CVD) risk factors, namely obesity, abnormal blood lipid profile, hypertension and insulin resistance have their roots in childhood and tend to track into adulthood.

### Innovations and breakthroughs

Deranged adiposity, blood lipid profile and other risk factors for CVD in celiac disease (CD) are still debated, and clear conclusions have yet to be reached. The authors' study aimed to describe CVD risk factors in a population of celiac children adhering to a gluten-free diet (GFD) for at least one year, in two Mediterranean countries.

### Applications

The authors' results suggest the importance of screening for CVD risk factors in celiac children both at diagnosis and during follow-up. Furthermore, they highlight that dietary counseling over time should target obesity and CVD risk factors in addition to monitoring adherence to a GFD in children and adolescents

diagnosed with CD.

### Terminology

The homeostatic model assessment is a method used to quantify insulin resistance and beta-cell function. This model correlated well with estimates using the euglycemic clamp method ( $r = 0.88$ ).

### Peer review

The present study is an appreciable work in the sense that it explores a concept that was not given attention before. With changing lifestyle and environment are celiac children more exposed to cardiovascular risk factors than normal population it needs to be validated in further prospective studies. The results of study convince for metabolic screening in celiac disease children in follow up visits and hence initiate early intervention to prevent cardiovascular morbidity.

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## 5-ASA colonic mucosal concentrations resulting from different pharmaceutical formulations in ulcerative colitis

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### Abstract

**AIM:** To compare the mucosal concentrations of 5-aminosalicylic acid (5-ASA) resulting from different pharmaceutical formulations and analyse the influence of inflammation on the mucosal concentrations.

**METHODS:** The study included 130 inflammatory bowel disease (IBD) patients receiving 5-ASA as pH-dependent-release formulations (73 patients), time-dependent-release formulations (11 patients), or pro-drugs (18 patients). In addition, 28 patients were receiving topical treatment (2-4 g/d) with pH-dependent-release formulations. Endoscopic biopsies were obtained from the sigmoid region during the colonoscopy. The 5-ASA concentrations (ng/mg) were measured in tissue homogenates

using high-pressure liquid chromatography with electrochemical detection. The *t* test and Mann-Whitney test, when appropriate, were used for statistical analysis.

**RESULTS:** Patients receiving pH-dependent-release formulations showed significantly higher mucosal concentrations of 5-ASA ( $51.75 \pm 5.72$  ng/mg) compared with patients receiving pro-drugs ( $33.35 \pm 5.78$  ng/mg,  $P = 0.01$ ) or time-dependent-release formulations ( $38.24 \pm 5.53$  ng/mg,  $P = 0.04$ ). Patients with endoscopic remission had significantly higher mucosal concentrations of 5-ASA than patients with active disease ( $60.14 \pm 7.95$  ng/mg vs  $35.66 \pm 5.68$  ng/mg,  $P = 0.02$ ). Similar results were obtained when we compared patients with the histological appearance of remission and patients with active histological inflammation ( $67.53 \pm 9.22$  ng/mg vs  $35.53 \pm 5.63$  ng/mg,  $P < 0.001$ ). Significantly higher mucosal concentrations of 5-ASA were detected in patients treated with both oral and topical treatments in combination compared with patients who received oral treatment with pH-dependent-release formulations alone ( $72.33 \pm 11.23$  ng/mg vs  $51.75 \pm 5.72$  ng/mg,  $P = 0.03$ ).

**CONCLUSION:** IBD patients showed significant variability in mucosal 5-ASA concentrations depending on the type of formulation, and the highest mean concentration was achieved using pH-dependent-release formulations.

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**Key words:** 5-aminosalicylic acid; Inflammatory bowel diseases; Mucosal concentration

**Core tip:** We report on the concentrations of 5-aminosalicylic acid in the colonic mucosa of ulcerative colitis patients. Significant variations in concentration were observed that were dependent on the type of pharmaceutical formulation and the presence of active disease. Combined oral and topical therapy yielded higher tissue mesalamine concentrations. These differences should

be taken into account in treatment strategies, especially in view of the fact that mesalamine can induce mucosal healing in ulcerative colitis.

D'Inca R, Paccagnella M, Cardin R, Pathak S, Baldo V, Giron MC, Sturniolo GC. 5-ASA colonic mucosal concentrations resulting from different pharmaceutical formulations in ulcerative colitis. *World J Gastroenterol* 2013; 19(34): 5665-5670 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5665.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5665>

## INTRODUCTION

Mesalamine[5-aminosalicylic acid (5-ASA)]-containing formulations represent the first-line therapy for the treatment of mild to moderate active ulcerative colitis and the prevention of recurrence<sup>[1]</sup>. When 5-ASA is administered and is absorbed by the colonic epithelium, *N*-acetyltransferase 1 metabolises a large amount of the 5-ASA to *N*-Ac-5-ASA, an inactive metabolite that is secreted back into the intestinal lumen and excreted in the faeces<sup>[2]</sup>. Sulphasalazine (Salazopyrin EN) is a pro-drug composed of sulphapyridine and 5-ASA connected by an azo-bond. Salazopyrin is metabolised to sulphapyridine and 5-ASA by the bacterial azoreductases of the intestinal microbiota. Sulphapyridine is excreted in the urine after most of it is absorbed from the colon, acetylated in the liver, and conjugated with glucuronic acid. The main action of sulphapyridine is to carry the 5-ASA moiety to the colon while preventing its proximal absorption. Absorption through the colon is necessary for the efficacy of 5-ASA<sup>[3]</sup>. Side effects, such as nausea, heartburn, headache, anaemia, skin rashes, reversible abnormalities of sperm number and morphology, and, rarely, hepatitis and nephritis, occur primarily due to high plasma sulphapyridine concentrations, which can generally be detected in patients taking higher doses of sulphasalazine or in genetically predisposed individuals (slow acetylators)<sup>[4]</sup>. Alternative preparations include modified-release formulations (which are supplied with pharmacological coatings that dissolve at a given pH or in a time-dependent manner) and pro-drugs. In pro-drugs such as sulphasalazine, an azo-bond links 5-ASA molecules to a carrier molecule. Similar compounds include olsalazine and balsalazide. Olsalazine was the first formulation, and it contains two 5-ASA molecules linked by an azo-bond. Approximately 12%-16% of patients being treated with olsalazine may suffer from secretory diarrhoea<sup>[5-7]</sup>. Balsalazide consists of 5-ASA linked *via* an azo-bond to an internal carrier (4-aminobenzoyl- $\beta$ -alanine). This formulation is not systemically absorbed. Modified-release formulations include delayed-release formulations (which release 5-ASA along a pH gradient) and sustained-release formulations (which release 5-ASA over a specified time interval) that are targeted to release 5-ASA in the lower small intestine and right colon. The pH-sensitive acrylic resin coat of Eudragit dissolves when the luminal pH rises above a critical

value. Pentasa is a sustained-release formulation that is gradually released based on a time-controlled mechanism. It consists of ethylcellulose-coated microgranules from which mesalazine is released into the small and large intestine. Its ethylcellulose coating is a semi-permeable membrane that dissolves when hydrated<sup>[8]</sup>. Combination therapy with oral and topical mesalazine administration can achieve higher mucosal concentrations than oral treatment alone<sup>[9]</sup>. Several *in vitro* studies have established a direct dose-effect relationship between 5-ASA and most of its immuno-inflammatory targets<sup>[10]</sup>. Furthermore, *in vivo* studies have demonstrated that there are inverse relationships between mucosal 5-ASA concentrations and the endoscopic and histological scores and mucosal levels of sIL-2R (a marker of mucosal inflammation). Higher drug mucosal concentrations lead to lower disease activity<sup>[11]</sup>. It follows that inadequate mucosal concentrations will result in inadequate disease management, particularly in patients with Crohn's disease and especially for the prevention of post-operative recurrence<sup>[12]</sup>. As a result, we can state that the therapeutic efficacy of 5-ASA is directly related to its mucosal concentration. Nevertheless, a large degree of individual variability in mucosal mesalamine concentrations exists, which is possibly due to differences in intestinal behaviour, dosage, route of administration, and the severity of the colonic inflammation<sup>[13-16]</sup>.

In this study, we focused on different pharmaceutical formulations of 5-ASA.

## MATERIALS AND METHODS

### *Patients and endoscopic procedures*

The study included 130 consecutive ulcerative colitis patients (mean age 47.76 years, range 23-84 years; 81 men and 49 women) who were referred to the Department of Surgical, Oncological and Gastroenterological Sciences, Gastroenterology Unit on continuous oral 5-ASA treatment. The general characteristics of the patients are shown in Table 1. All of the patients were receiving treatment with oral 5-ASA three times per day in one of three different pharmaceutical formulations: pH-dependent delayed-release formulations (73 patients at a dose of 2.4 g daily; Asacol Giuliani-Bracco Italy, Pentacol Sofar Italy), mesalamine pro-drug (18 patients at a dose of 3 g daily; Salazopyrin EN, Pfizer, Italy), and time-dependent sustained-release formulations (11 patients at a dose of 3 g daily; Pentasa, Ferring, Italy). There were 28 patients who received both oral and topical (2-4 g/d by enema) pH-dependent-release formulations. The patients receiving combined treatment (mean age 46.5 years, range 23-79 years; 64.2% male) were comparable with respect to age and gender distribution to patients receiving oral therapy alone (mean age 47.24 years, range 25-84 years; 67.1% male). No concomitant immunological, renal, or hepatic disorders were reported by any of the patients. Moreover, none of the patients were taking steroids, immunosuppressive agents, antibiotics, H<sub>2</sub>-receptor antagonists, or proton pump inhibitors. Colonoscopy was performed for surveillance or to detect symptom re-exacerbation. Bowel

**Table 1** Demographic and clinical characteristics of patients

Characteristics	pH-dependent delayed release ( <i>n</i> = 73)	Pro-drugs ( <i>n</i> = 18)	Time-dependent sustained release ( <i>n</i> = 11)
Age (mean ± SE) (yr)	47.24 ± 1.61	51.38 ± 2.39	47.54 ± 4.97
Gender (M/F)	49/24	8/10	6/5
Extent of disease			
Proctosigmoiditis	22%	22%	0%
Left colitis	10%	11%	0%
Pancolitis	68%	67%	100%
Age at diagnosis (mean ± SE)	34.88 ± 1.61	32.72 ± 2.30	31.09 ± 3.30
Duration of disease (yr)	11.56 ± 0.81	17.22 ± 4.21	15.00 ± 2.62
Time since last 5-ASA administration (h)	21.43 ± 1.22	23.88 ± 4.21	20.63 ± 2.52

M: Male; F: Female; 5-ASA: 5-aminosalicylic acid.

cleansing was achieved using a polyethylene glycol oral solution, 3-5 L, on the day before the colonoscopy. After the patients provided their informed consent, the time at which they took their last pill/enema was recorded, and two biopsies were taken from the sigmoid region at 25 cm from the anal verge. The observation of inflammatory changes in the colonic mucosa on endoscopy was considered endoscopic activity following the Baron classification, while the absence of mucosal inflammatory changes was considered endoscopic remission<sup>[17,18]</sup>. The histological activity of the disease was examined according to a semi-quantitative score that took into consideration the extent of lymphocytic and polymorphonuclear leukocyte infiltration, mucus depletion, crypt distortion, the presence of crypt abscesses, and lymphoid follicle activation. Histological remission was defined as the absence of inflammatory changes in the mucosa<sup>[19]</sup>.

### Chemicals and reagents

Purified 5-ASA was obtained from Acros (NJ, United States). Purified water and methanol were used for the high-pressure liquid chromatography (HPLC) analysis. The use of these products was important to reduce background current and noise within the HPLC-electrochemical detection system.

A stock solution of 5-ASA was prepared at 1 mg/mL in 0.2 mol/L protocatechuic acid (PCA), 100 μmol/L ethylenediaminetetraacetic acid (EDTA), and 100 μmol/L sodium metabisulfite and stored at 4 °C.

### Tissue preparation

The specimens were homogenised in 0.2 mol/L PCA, 100 μmol/L EDTA, and 100 μmol/L sodium metabisulfite at 4 °C and then centrifuged (1800 *g*) for 10 min. The supernatants were collected and filtered with 0.2-μm cellulose acetate filters and stored at -80 °C. A sample volume of 20 μL was used throughout this study.

### Quantification of 5-ASA

A sensitive HPLC method capable of measuring the mucosal 5-ASA concentrations was used. Briefly, analyses were performed on a chromatographic apparatus (Alliance Waters, United States) that consisted of a model 2695 solvent-delivery system and an electrochemical detector, Coulochem (ESA, United States) Model 5100A,

that was integrated with Empower Software (Waters, United States).

Separation of the analytes was achieved using a reversed-phase DHBA-250 column (5 μm, 250 × 3.0 mm), and the analytes were detected on a high-sensitivity analytical cell model 5011, with the oxidation potentials of electrodes 1 and 2 adjusted to +750 mV to oxidise the 5-ASA.

The mobile phase consisted of 50 mmol/L sodium acetate, 50 mmol/L sodium citrate, 8% methanol, and 2% 2-propanol. The pH of the mobile phase was adjusted to 2.5 with phosphoric acid after the addition of the organic modifiers. The mobile phase was passed through the system at 0.5 mL/min.

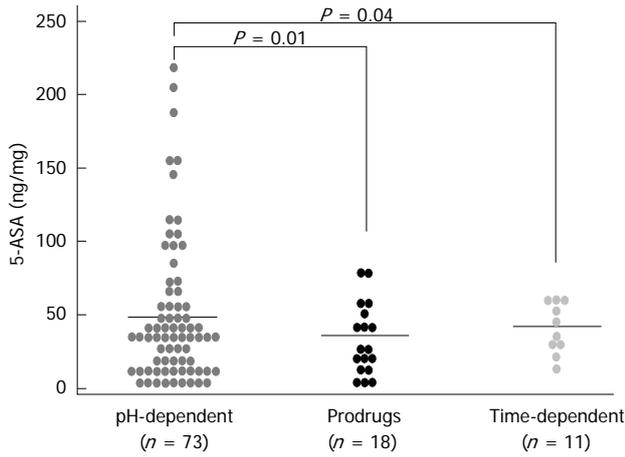
The standard curve for 5-ASA was linear in the selected range ( $r^2 = 0.99$ ) with an inter-assay coefficient of variation < 4%.

### Statistical analysis

The mucosal 5-ASA concentrations in patients being treated with any pharmaceutical 5-ASA formulation and in patients with different endoscopic and histological degrees of activity were compared using an unpaired Student's *t* test and the Mann-Whitney test where appropriate. A *P* value of 0.05 or less was considered significant. The data are presented as the mean ± SE. The 5-ASA concentrations are expressed as ng/mg tissue. The statistical analyses were performed using the statistical software package SPSS for Windows, version 13.0 (SPSS, Chicago, IL, United States).

## RESULTS

The demographic and clinical characteristics of the patients being treated with different pharmaceutical formulations were similar (Table 1). Figure 1 shows the distribution of the mucosal concentrations of mesalamine in the sigmoid region for the three groups studied. The mean mucosal mesalamine concentration was significantly higher in patients being treated with pH dependent-release formulations than in patients being treated with pro-drugs (51.75 ± 5.72 ng/mg *vs* 33.35 ± 5.78 ng/mg, *P* = 0.01). Similarly, the concentration of mesalamine was significantly higher in patients being treated with pH-dependent-release formulations than in patients being

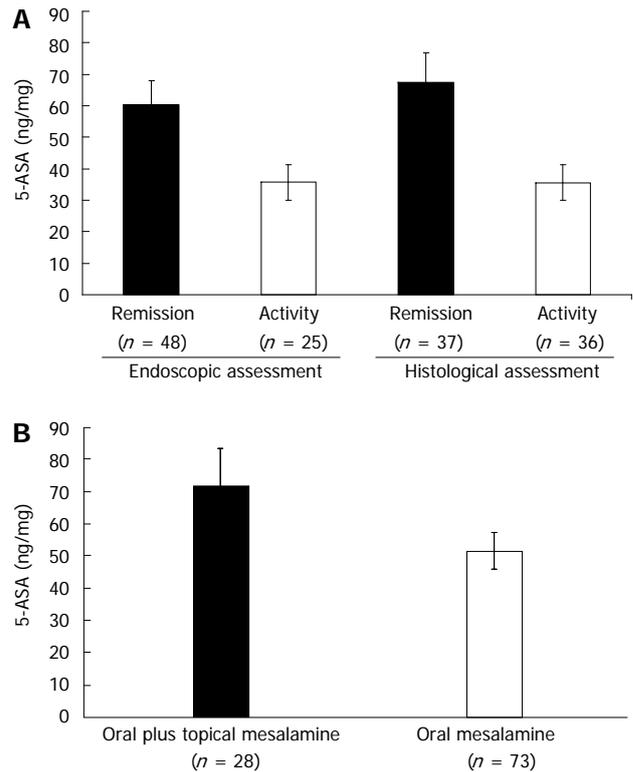


**Figure 1** Distribution of 5-aminosalicylic acid mucosal concentrations in the sigmoid colon in patients receiving oral 5-aminosalicylic acid either pH-dependent release formulations, time-dependent release formulations or prodrugs. 5-ASA: 5-aminosalicylic acid.

treated with time-dependent-release formulations ( $51.75 \pm 5.72$  ng/mg *vs*  $38.24 \pm 5.53$  ng/mg,  $P = 0.04$ ). Furthermore, the absolute mucosal 5-ASA concentrations were significantly higher in patients being treated with pH-dependent-release formulations; specifically, 28% of the patients were found to have mucosal 5-ASA concentrations above 70 ng/mg of tissue, which was the highest concentration achieved with any of the other formulations. Figure 2A shows the distribution of the mucosal mesalamine concentrations in patients receiving pH-dependent-release formulations according to endoscopic and histological activity or disease remission.

Twenty-five patients showed active disease in the sigmoid colon, while the remaining 48 patients presented an endoscopic appearance of remission or a normal assessment. The histological grade of the mucosal inflammation in the sigmoid colon was “active” in 36 patients, while the remaining 37 patients presented a “normal” histological assessment or the appearance of remission. Patients with a normal endoscopic assessment or an endoscopic appearance of remission showed significantly higher concentrations of mucosal 5-ASA than patients with active endoscopic inflammation ( $60.14 \pm 7.95$  ng/mg *vs*  $35.66 \pm 5.68$  ng/mg,  $P = 0.02$ ). Similarly, significant differences were found in the mucosal 5-ASA concentrations between patients with a normal histological assessment or the histological appearance of remission and patients with active histological inflammation ( $67.53 \pm 9.22$  ng/mg *vs*  $35.53 \pm 5.63$  ng/mg,  $P < 0.001$ ).

Twenty-eight patients received both pH-dependent mesalamine orally at a dose of 2.4 g/d and rectal mesalamine at a dose of 4 g/d. Figure 2B shows the distribution of mucosal 5-ASA concentrations in patients receiving combination treatment compared with those in patients receiving oral treatment alone. The mean mucosal 5-ASA concentration was significantly higher in patients receiving combined oral and topical treatment than in patients taking oral mesalamine only ( $72.33 \pm 11.23$  ng/mg *vs*



**Figure 2** 5-aminosalicylic acid mucosal concentrations in the sigmoid colon. A: In patients receiving oral 5-aminosalicylic acid (5-ASA) pH-dependent release formulations according to endoscopic and histological grading of disease; B: In patients receiving 5-ASA pH-dependent release formulations on oral plus topical treatment or oral therapy alone.

$51.75 \pm 5.72$  ng/mg,  $P = 0.03$ ).

## DISCUSSION

IBD patients usually receive chronic treatment with 5-ASA to both control the active disease and reduce the frequency and severity of clinical relapses. Although this drug has been used for the last 50 years, the precise mechanism of action of 5-ASA, with the exception of its topical efficacy, is unknown<sup>[20]</sup>. However, its topical activity implies the necessity of its delivery to the inflamed tissue. Therefore, appropriate dosages and targeted delivery of the drug are needed. Many studies have demonstrated marked variability in 5-ASA metabolism and distribution following oral dosing<sup>[14,15]</sup>. Thus, the clinical course of the disease, which encompasses periods of prolonged remission and frequent episodes of relapse, could derive from variable availability of the drug. We know from several *in vitro* studies that there is a direct relationship between the 5-ASA concentration and its therapeutic efficacy<sup>[21,22]</sup>. Previous *in vivo* studies have reported that the therapeutic efficacy is dose-dependent. In fact, high mucosal mesalamine concentrations have been shown to be associated with endoscopic and histological scores of disease remission or mild activity rather than moderate or severe disease in ulcerative colitis. They have also been shown to be associated with a reduced risk of severe post-operative recurrences in Crohn’s disease<sup>[11,12]</sup>. However, the appro-

priate mucosal concentration is unknown, and it is therefore not easy to provide guidance. There is wide inter-individual variability in mucosal concentrations, and the factors governing tissue drug concentrations are largely unknown because increased mucosal concentrations do not always derive from increased oral doses<sup>[23]</sup>.

Adherence to therapy may be another important factor that influences mucosal concentrations. We did not test adherence specifically; however, samples were obtained only from patients who reported that they had taken their last pill within 24 h of endoscopy. Moreover, the 5-ASA concentration can be variable along the entire length of the colon. Oral administration of 5-ASA ensures a higher drug concentration in the right colon than in the rectum, where the amount often becomes negligible; however, the rectum is the preferential site of the disease and is almost invariably affected. Because the oral dose is not strictly related to the 5-ASA concentration, the tissue absorption, drug metabolism and excretion, and pharmaceutical variables, such as the route of administration and formulation type, need to be investigated. Several clinical studies have confirmed that the highest therapeutic efficacy is reached when patients are treated with oral and topical treatments<sup>[24,25]</sup>. Patients with active distal disease in whom oral treatment is frequently inadequate respond to mesalazine enemas<sup>[9]</sup>. Rectal formulations are also successful in maintaining remission in patients suffering from frequent relapses. According to Frieri *et al.*<sup>[26]</sup>, patients being treated with oral and topical treatments show similar mucosal mesalamine concentrations in the rectum and in the descending colon, while oral treatment alone results in a higher drug concentration in the descending colon than in the rectum. Indeed, we found higher mucosal concentrations in the sigmoid mucosa in patients receiving both topical and oral 5-ASA than in patients receiving oral mesalamine alone. It is possible that the level of adherence may have been higher in patients experiencing active disease who therefore were receiving combined therapy.

To date, few and discordant reports have investigated the relationship between the colonic mucosal concentration of 5-ASA and its different pharmaceutical formulations<sup>[27,28]</sup>. We demonstrated that the sigmoid mucosal concentration of 5-ASA was significantly higher in IBD patients receiving pH-dependent delayed-release formulations compared with patients receiving preparations dependent on bacterial degradation (pro-drugs). Similarly, the mucosal concentration of 5-ASA in the sigmoid mucosa of the pH-dependent delayed-release formulations group was higher than that of the time-dependent sustained-release formulations. Moreover, we found that the absolute mucosal 5-ASA concentrations were significantly higher in patients being treated with pH-dependent-release formulations than in patients being treated with pro-drugs. In fact, in 28% of the patients receiving pH-dependent-release formulations, the mucosal 5-ASA concentration was above 70 ng/mg of tissue, which was the highest value achieved from any of the formulations. Because of the dose-related anti-inflammatory effect

of 5-ASA, we should expect the highest efficacy when the highest mucosal tissue concentration of 5-ASA is achieved. As reported by Hussain *et al.*<sup>[23]</sup>, rectal mucosal concentrations of aminosaliclates are lower during relapses. As previously demonstrated by Frieri *et al.*<sup>[11]</sup>, we confirmed that the colonic mucosal concentrations of 5-ASA were inversely related to disease activity as measured by both endoscopic and histological evaluation. Oedema occurring during the active phase of the disease may account for a possible dilution effect on the biopsy specimens. Alternatively, the faster rate of tissue renewal in the presence of inflammation could produce a wash-out effect on the drug and contribute to a reduced mucosal mesalamine concentration.

In conclusion, because we have reinforced the relationship between tissue mesalamine levels and disease activity, additional studies are needed to determine how to reach optimal mucosal concentrations of 5-ASA. We have demonstrated that different pharmaceutical preparations achieve different mucosal concentrations and that disease activity lowers the drug mucosal concentration. Higher dosages could therefore be justified during active disease to obtain better clinical results.

## COMMENTS

### Background

The use of mesalamine represents the first-line treatment strategy in patients with ulcerative colitis. Many formulations are available, and they are often used interchangeably because it is assumed that they are all equally effective.

### Research frontiers

The mucosal concentration of 5-aminosalicylic acid (5-ASA) was measured in the colon of ulcerative colitis patients and at the anastomotic site of Crohn's disease patients in the post-operative setting. In this study, the authors demonstrated that tissue concentrations could be measured by high-pressure liquid chromatography and that the type of 5-ASA formulation influenced the mucosal concentration.

### Innovations and breakthroughs

Mesalamine is poorly absorbed; therefore, blood monitoring is not helpful. The tissue concentration may represent a better tool for tailoring therapy in ulcerative colitis patients.

### Applications

The response to treatment with mesalamine in ulcerative colitis patients can be optimised by administering the proper formulation of the drug at the right dose to the patient.

### Peer review

The authors examined the colonic mucosal concentrations of mesalamine in patients with ulcerative colitis who were being treated with different formulations of mesalamine. The results are interesting and may guide clinicians in tailoring therapies to their patients.

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## Eviendep<sup>®</sup> reduces number and size of duodenal polyps in familial adenomatous polyposis patients with ileal pouch-anal anastomosis

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Author contributions: Calabrese C initiated the study, coordinated the conduct of the whole study, performed the endoscopies; Calabrese C, Praticò C and Calafiore A prepared the draft of the manuscript; Calabrese C and Rizzello F re-evaluated all the endoscopy videos and photos in a blinded manner and scored the images separately; Coscia M, Gentilini L and Poggioli G performed the surgical procedures; Gionchetti P and Campieri M reviewed the manuscript; all authors approved the final version of the manuscript.

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### Abstract

**AIM:** To evaluate if 3 mo oral supplementation with Eviendep<sup>®</sup> was able to reduce the number of duodenal polyps in familial adenomatous polyposis (FAP) patients with ileal pouch-anal anastomosis (IPAA).

**METHODS:** Eleven FAP patients with IPAA and duodenal polyps were enrolled. They underwent upper gastrointestinal (GI) endoscopy at the baseline and after 3 mo of treatment. Each patient received 5 mg Eviendep twice a day, at breakfast and dinner time, for 3 mo. Two endoscopists evaluated in a blinded manner the number and size of duodenal polyps. Upper GI endoscopies with biopsies were performed at the baseline

(T0) with the assessment of the Spigelman score. Polyps > 10 mm were removed during endoscopy and at the end of the procedure a new Spigelman score was determined (T1). The procedure was repeated 3 mo after the baseline (T2). Four photographs were examined for each patient, at T1 and T2. The examined area was divided into 3 segments: duodenal bulb, second and third portion duodenum. Biopsy specimens were taken from all polyps > 10 mm and from all suspicious ones, defined by the presence of a central depression, irregular surface, or irregular vascular pattern. Histology was classified according to the updated Vienna criteria.

**RESULTS:** At baseline the mean number of duodenal detected polyps was 27.7 and mean sizes were 15.8 mm; the mean Spigelman score was 7.1. After polypectomy the mean number of duodenal detected polyps was 25.7 and mean sizes were 7.6 mm; the mean Spigelman score was 6.4. After 3 mo of Eviendep *bid*, all patients showed a reduction of number and size of duodenal polyps. The mean number of duodenal polyps was 8 ( $P = 0.021$ ) and mean size was 4.4 mm; the mean Spigelman score was 6.6. Interrater agreement was measured. Lesions > 1 cm found a very good degree of concordance ( $\kappa$  0.851) and a good concordance was as well encountered for smaller lesions ( $\kappa$  0.641).

**CONCLUSION:** Our study demonstrated that short-term (90 d) supplementation with Eviendep<sup>®</sup> in FAP patients with IPAA and with recurrent adenomas in the duodenal mucosa, resulted effective in reducing polyps number of 32% and size of 51%.

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**Key words:** Familial adenomatous polyposis; Ileal pouch-anal anastomosis; Duodenal polyps; Eviendep

**Core tip:** Our open study demonstrated for the first time that short-term (90 d) supplementation with Eviendep® in familial adenomatous polyposis patients with ileal pouch-anal anastomosis and with recurrent adenomas in the duodenal mucosa, resulted effective in reducing polyps number of 32% and size of 51%. Eviendep® was easy to manage and its daily use was well tolerated by the patients. Its safety was guaranteed by its composition. Each ingredient is blended into the composition in a lower dose than the one otherwise needed for the single component to similarly exert the desired effect, thus leading to synergistic and/or potentiating effect, with the added advantage of higher safety, even over long-term exposure.

Calabrese C, Praticò C, Calafiore A, Coscia M, Gentilini L, Poggioli G, Gionchetti P, Campieri M, Rizzello F. Eviendep® reduces number and size of duodenal polyps in familial adenomatous polyposis patients with ileal pouch-anal anastomosis. *World J Gastroenterol* 2013; 19(34): 5671-5677 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5671.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5671>

## INTRODUCTION

Familial adenomatous polyposis (FAP) is a disease with autosomal dominant inheritance. It is caused by an alteration of the *FAP* gene that is located on chromosome 5q21, affecting roughly 1 in 15000 live births in the Northern European population. FAP shares its phenotype with biallelic Homolog Gene Mutation Carriers, characterized by the early onset of hundreds to thousands of adenomas throughout the colon, with a nearly 100% progression to colorectal cancer by the age of 35-45 years in untreated subjects<sup>[1-3]</sup>. Patients with FAP have a cumulative lifetime risk of over 80% of developing duodenal adenomas, the precancerous lesions of duodenal adenocarcinoma. Consequently, these patients have a 4% lifetime risk of peri-ampullary or duodenal adenocarcinoma<sup>[4,5]</sup>.

Early prophylactic colorectal surgery changed the prognosis of patients with FAP, and nowadays desmoids and peri-ampullary duodenal cancers are the most common causes of death in these patients<sup>[6,7]</sup>.

Nearly 100% of FAP patients will develop duodenal adenomatosis<sup>[5-11]</sup> with an estimated lifetime risk of progression to duodenal carcinoma of 5%-10%<sup>[5-12]</sup>. The severity of duodenal adenomatosis is graded according to the Spigelman classification<sup>[10]</sup>, which ranges from grade 0 to IV and is based on the number, size and histopathological features of the duodenal adenomas. Patients with advanced Spigelman stages are most at risk of developing duodenal carcinoma<sup>[5,10]</sup>. Current guidelines recommend frequent endoscopic surveillance in these patients, which improved the prognosis through earlier detection of duodenal malignancy<sup>[13]</sup>.

A new recent line of intervention focuses on the role of the estrogen receptors (ERs) in intestinal carcinogenesis<sup>[14-16]</sup>. A pivotal role of ER-β has been suggested in preventing malignant transformation of colon epithelial cells in humans<sup>[16]</sup>. Data confirm the involvement of ERs-β in colorectal carcinogenesis and suggest a possible explanation for the protective effect of estrogens in cancer development<sup>[17-19]</sup>. They also provided further support of the role of vegetable-rich diets in the prevention of bowel cancer, thanks to their high content of phytoestrogens. Phytoestrogens include a variety of vegetable derived compounds with estrogen-like chemical structure and differential selectivity to the two ERs, ERα and ERβ. Particularly the dietary flavonolignan silymarin and the lignans have been reported to exert selective agonism to the ERβ the former, and preferential selectivity the latter. Since the promotion and progression of carcinogenesis are susceptible to nutritional interventions<sup>[5]</sup>, the aim of this study was to evaluate if 3 mo oral supplementation with a patented blend of phytoestrogens and indigestible and insoluble fibres (Eviendep®, CM&D Pharma Limited, United Kingdom) was able to reduce the number of duodenal polyps in FAP patients with ileal pouch-anal anastomosis (IPAA).

## MATERIALS AND METHODS

### Population

This study was conducted in FAP patients with IPAA. The patients were ongoing the surveillance program at our department by screening upper gastrointestinal (GI) endoscopies for the follow-up of duodenal adenoma (polyp) recurrence and progression to adenocarcinoma. Cardiovascular diseases and inadequate organ function were study exclusion criteria. All patients gave their informed consent.

### Endoscopic and histological procedures

Endoscopies were performed after an overnight fast; patients were prepared by a light sedation (*iv* midazolam coupled with 20 mg of scopolamine N-butyl bromide) and were examined with an upper GI endoscopy (Olympus GIF 165) until the third portion of the duodenum.

The severity of duodenal polyposis was classically assessed using the Spigelman classification<sup>[10]</sup>. This classification system describes five stages in duodenal polyposis development. Points are accumulated for number, size and histology of adenomatous polyps. Spigelman stage I (1-4 points) indicates mild disease, whereas stage III-IV (> 6 points) implies severe duodenal polyposis. The traditional Spigelman classification classified adenomas into mild, moderate and severe dysplasia, whereas the updated classification distinguishes low- and high-grade dysplasia.

Upper GI endoscopies with biopsies were performed by the first operator (Calabrese C) at the baseline (T0) with the assessment of the Spigelman score. Polyps > 10 mm were removed during endoscopy and at the end

of the procedure a new Spigelman score was determined (T1). The procedure was repeated 3 mo after the baseline (T2), which also coincided with the 3 mo oral supplementation of Eviendep.

The first operator together with another experienced endoscopist (Rizzello F) re-evaluated all the endoscopy videos and photos. They evaluated images in a blinded manner and scored the images separately. Each expert first evaluated them individually and then in case of disagreement, a consensus was reached afterward by discussion.

Four photograms were examined for each patient, at T1 and T2. The examined area (photogram) was divided into 3 segments: duodenal bulb, second and third portion duodenum. For each segment the two operators were asked to assess the total number of polyps observed and their sizes by using an open biopsy forceps (8 mm).

Lastly, biopsy specimens were taken from all polyps > 10 mm and from all suspicious ones, defined by the presence of a central depression, irregular surface, or irregular vascular pattern.

Histologic samples were processed by using standard procedures and evaluated by gastroenterology specialized pathologists. Histology was classified according to the epithelium type (tubular, tubulovillous, or villous adenoma) and the degree of dysplasia (none, low grade, high grade, or cancer according to the updated Vienna criteria).

### Treatment procedures

Eviendep<sup>®</sup> was chosen for its specifically high content of phytoestrogens and fibres. It comprises the selective ER $\beta$ -targeted flavonolignan silymarin (qualified for a 30% content in silibinin) and lignans (qualified for at least 40% of secoisolariciresinol diglucoside), in combination with non-starch, insoluble and indigestible fibres (qualified for or less than 5% lignin content). Each patient received 5 mg Eviendep twice a day, at breakfast and dinner time, for 3 mo.

### Statistical analysis

Statistical significance was determined using Student's *t*-tests for paired and unpaired samples. Treatment results were compared by  $\chi^2$  test for comparison of proportion with a 95% confidence interval (CI). All statistical analyses were 2-tailed, and significance was accepted at a *P* value < 0.05. To test the reproducibility of these findings, interrater agreement was calculated with kappa analysis. A score of < 0.20 was considered poor, 0.21 to 0.40 fair, 0.41 to 0.60 moderate, 0.61 to 0.80 good, and 0.81 to 1.00 very good. We performed all the statistical analyses using a statistical software package (SPSS Inc, Chicago, IL, United States).

## RESULTS

Eleven patients (M/F 5/6; mean age 40.7 years, SD  $\pm$

**Table 1 Findings at baseline and patients characteristics in 11 jejunal polyposis patients**

Patient No.	Age, yr (gender)	Age at colectomy (yr)	No. of duodenal polyps	Max size of duodenal polyps (mm)	Spigelman score
1	32 (F)	18	53	12	8
2	57 (M)	31	32	21	8
3	43 (F)	21	21	22	8
4	31 (F)	16	20	5	7
5	23 (M)	22	30	12	7
6	29 (M)	18	12	5	6
7	62 (M)	32	19	23	7
8	31 (F)	17	22	19	7
9	45 (M)	28	19	12	6
10	35 (F)	19	54	21	7
11	60 (F)	31	23	22	7

F: Female; M: Male.

13.6 years) met the inclusion criteria and were enrolled; they were followed prospectively between November 2012 and January 2013 at our department outpatients' clinic. The mean age at colectomy was  $23 \pm 6.2$  years and mean age of IPAA was  $17.7 \pm 8.5$  years. Table 1 shows the demographic, clinical characteristics and polyps' histological features.

At baseline (T0) the mean number of duodenal detected polyps was  $27.7 \pm 13.8$  (range 12-54) and mean sizes were  $15.8 \pm 6.8$  mm (range 5-23 mm); the mean Spigelman score was  $7.1 \pm 0.7$  (range 6-8). Histology confirmed tubular adenomatous tissue with low-grade dysplasia.

After polypectomy (T1) the mean number of duodenal detected polyps was  $25.7 \pm 13.4$  (range 10-51) and mean sizes were  $7.6 \pm 1.9$  mm (range 5-10 mm); the mean Spigelman score was  $6.4 \pm 0.5$  (range 6-7). After 3 mo of Eviendep *bid* (T2), all patients showed a reduction of number and size of duodenal polyps. The mean number of duodenal polyps was  $8 \pm 6.2$  (range 2-19) ( $\chi^2 = 28.42$ , *P* = 0.021) and mean size was  $4.4 \pm 2.1$  mm (range 2-9 mm); the mean Spigelman score was  $6.6 \pm 0.7$  (range 4-6) (Table 2, Figure 1).

Interrater agreement was measured. Lesions > 1 cm found a very good degree of concordance (kappa value 0.851) and a good concordance was as well encountered for smaller lesions (kappa value 0.641).

All the eleven patients completed the study. Compliance was excellent; only one patient reported mild intestinal bloating, and the therapy was not discontinued.

## DISCUSSION

Colonic surveillance programs and proctocolectomy with IPAA have improved the prognosis of patients with FAP. Current leading disease-related causes of death are desmoids tumours and duodenal adenocarcinomas. Duodenal cancer is nowadays the most important cause of death in FAP patients<sup>[13]</sup>. With respect to the duodenal manifestation of this disease, surveillance and prophylactic treatment strategies will hopefully further improve

**Table 2 Findings at baseline after polypectomies and after 3 mo of treatment**

Patient No.	T1			T2		
	Duodenal polyps (n)	Max size of duodenal polyps (mm)	Spigelman score	Duodenal polyps (n)	Max size of duodenal polyps (mm)	Spigelman score
1	50	10	7	19	4	5
2	30	8	7	10	8	6
3	20	9	6	4	9	5
4	18	5	6	2	3	4
5	29	8	7	19	4	5
6	10	5	6	4	2	4
7	18	8	6	10	4	5
8	19	10	6	4	4	4
9	17	8	6	2	3	4
10	51	8	7	10	4	5
11	21	5	7	4	4	4

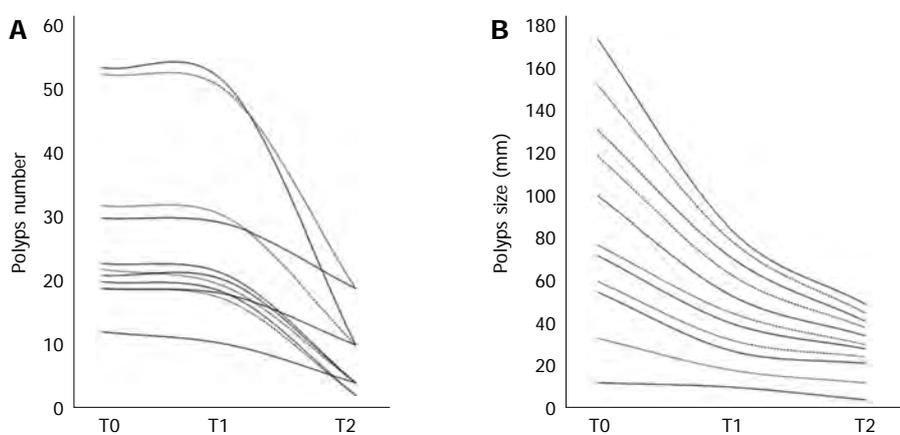


Figure 1 Changes in total polyps number and max size in all patients at baseline (T0), after polypectomy (T1) and after 3 mo of treatment (T2). A: Polyps number; B: Polyps size.

prognosis. The secondary chemoprevention of duodenal cancer identifies three different lines of intervention.

**First chemopreventive strategy**

Pharmacological intervention studies have been primarily focused on targeting the inflammatory pathway of cyclooxygenase-2 (COX-2), an enzyme with increased expression in experimental and human intestinal neoplasia. Most of these studies tested the efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid on either adenoma regression or new polyps prevention<sup>[20]</sup>. Randomized placebo-controlled trials have demonstrated that the NSAIDs sulindac and the selective COX-2 inhibitors celecoxib and rofecoxib have chemopreventive efficacy in FAP, with a significant regression of polyps<sup>[21-23]</sup>.

However, evaluations of the safety of the COX-2 inhibitors and NSAIDs showed an elevated risk of serious cardiac disorders, selected renal and hypertension events and a rebound effect on adenomas after the discontinuation of the treatments<sup>[24]</sup>.

**Second chemopreventive strategy**

Dietary interventions are the second chemopreventive treatment line. The studies on these interventions are based on potential chemopreventive properties of several dietary components and on the evidence of epigenetic mutations of tumour suppressor genes in the intestinal

mucosa after an unbalanced diet<sup>[25,26]</sup>. These studies forecasted an increase in servings/day of either fruit, vegetables, wholegrain and fibres in populations at risk of colorectal carcinoma, as well as the supplementation of specific nutrient blends.

**Third chemopreventive strategy**

The third new line of intervention focuses on the role of the ERs<sup>[27]</sup>. Since the discovery of ERs in the colonic tumour cells<sup>[28,29]</sup>, several epidemiological and clinical studies have supported the idea that estrogens play a protective role in the pathogenesis of colorectal neoplastic lesions, suggesting their potential use in the prevention of colorectal cancer<sup>[16,30-33]</sup>. There is evidence of estrogens proliferative modulation not only on the usual estrogens responsive tissues<sup>[34]</sup> but also on other apparatuses<sup>[35]</sup>.

Estrogens bind two types of receptors: estrogen receptor-alpha (ER- $\alpha$ ), prevalent in the breast, bone, cardiovascular tissue, urogenital tract and central nervous system, and estrogen receptor-beta (ER- $\beta$ ), prevalent in the gut<sup>[36,37]</sup>.

ER- $\beta$  expression is significantly lower in colonic adenocarcinoma cells than in normal colonic epithelial cells and this reduction is directly correlated with the degree of tumour dedifferentiation<sup>[15]</sup>. Although ER expression has been widely investigated in colorectal cancer cells (CRCs)<sup>[15,38,39]</sup>, few data are available about colorectal pre-

cancerous lesions.

Recently data confirm the involvement of ERs- $\beta$  in colorectal carcinogenesis and suggest a possible explanation for the protective effect of oestrogens on cancer development<sup>[17-19]</sup>. They also further support the role of vegetable-rich diets in the prevention of bowel cancer, thanks to their high content of phytoestrogens<sup>[5]</sup>.

In particular milk thistle, traditionally used as an antioxidant and antifibrotic agent in chronic liver disease<sup>[14]</sup>, is the source of silymarin, an ER- $\beta$  selective-agonist<sup>[15]</sup>. Silymarin has been documented to be an effective chemopreventive in the intestinal tumour progression<sup>[15,16,39]</sup>. The lignans, non-soluble dietary fibres has been reported to be similarly effective in the chemoprevention of CRC<sup>[17,40]</sup> most likely for their ability to absorb potential carcinogens in the intestinal lumen<sup>[17-18]</sup>. Barone *et al*<sup>[41]</sup> demonstrated with animal studies that ER- $\beta$  expression is amenable to dietary modulation to regress and/or oppose the progression of the adenoma-adenocarcinoma sequence. In a randomized, double-blind and placebo controlled study in patients undergoing surveillance colonoscopy because of recurrent sporadic adenomatous polyposis, a two months supplementation of Eviendep<sup>®</sup> (5 g *bid*) was able to specifically induce the expression of the ER $\beta$  in the colon mucosa, with optimal tolerability and safety<sup>[42]</sup>. The same group recently demonstrated that the oral supplementation of Eviendep to patients undergoing surveillance colonoscopy because of recurrent sporadic adenomatous polyposis was able to significantly increase the expression of ER- $\beta$  in the colon mucosa, with optimal tolerability and safety<sup>[42]</sup>.

At the same time, Yamada *et al*<sup>[43]</sup> performed a study with capsule endoscopy and found that patients with duodenal polyps had a larger number of polyps in the small intestine than those without duodenal polyps. In our experiences 8 of the 11 patients enrolled were previously investigated by capsule endoscopy. In our subset of patients there was no evidence of polyps in the small intestine.

In conclusion, our study demonstrated for the first time that short-term (90 d) supplementation with Eviendep<sup>®</sup> in FAP patients with IPAA and with recurrent adenomas in the duodenal mucosa, resulted effective in reducing polyps number by 32% and size by 51%. Eviendep<sup>®</sup> was easy to manage and its daily use was well tolerated by the patients. Its safety was guaranteed by its composition. Each ingredient is blended into the composition in a lower dose than the one otherwise needed for the single component to similarly exert the desired effect, thus leading to synergistic and/or potentiating effect, with the added advantage of higher safety, even over long-term exposure. Further molecular studies are needed to better confirm the role of estrogens in the duodenal mucosa, but we do believe that they also play a central role in duodenal carcinogenesis in the colon.

## COMMENTS

### Background

Familial adenomatous polyposis (FAP) is a disease with autosomal dominant

inheritance. Early prophylactic colorectal surgery changed the prognosis of patients with FAP, and duodenal cancer is nowadays the most important cause of death in FAP patients.

### Research frontiers

The recent discover of the involvement of estrogen receptors (ERs)- $\beta$  in colorectal carcinogenesis suggests a possible explanation for the protective effect of estrogens in cancer development. This also provided further support of the role of vegetable-rich diets in the prevention of bowel cancer, thanks to their high content of phytoestrogens (ER- $\beta$  selective agonists).

### Innovations and breakthroughs

Several epidemiological and clinical studies have supported the idea that estrogens play a protective role in the pathogenesis of colorectal neoplastic lesions, suggesting their potential use in the prevention of colorectal cancer. The aim of this study was to evaluate if dietary supplementation with phytoestrogens, selective agonists of the estrogen receptor, was able to prevent as well the progression of carcinogenesis in duodenal polyps.

### Applications

Their study demonstrated that short-term supplementation with phytoestrogens in FAP patients with ileal pouch-anal anastomosis (IPAA) is effective in reducing duodenal polyps number and size.

### Peer review

The authors investigated the effects of short-term (90 d) supplementation with Eviendep on the reduction of the number and size of duodenal polyps in FAP patients who had undergone IPAA.

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## Fatty acids of erythrocyte membrane in acute pancreatitis patients

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### Abstract

**AIM:** To evaluate changes in the fatty acid composition of erythrocyte membrane phospholipids during severe and mild acute pancreatitis (AP) of alcoholic and nonalcoholic etiology.

**METHODS:** All consecutive patients with a diagnosis of AP and onset of the disease within the last 72 h admitted to the Hospital of Lithuanian University of Health Sciences between June and December 2007 were included. According to the Acute Physiology and Chronic

Health Evaluation (APACHE II) scale, the patients were subdivided into the mild (APACHE II score < 7,  $n = 22$ ) and severe (APACHE II score  $\geq 7$ ,  $n = 17$ ) AP groups. Healthy individuals ( $n = 26$ ) were enrolled as controls. Blood samples were collected from patients on admission to the hospital. Fatty acids (FAs) were extracted from erythrocyte phospholipids and expressed as percentages of the total FAs present in the chromatogram. The concentrations of superoxide dismutase and glutathione peroxidase were measured in erythrocytes.

**RESULTS:** We found an increase in the percentages of saturated and monounsaturated FAs, a decrease in the percentages of total polyunsaturated FAs (PUFAs) and  $n-3$  PUFAs in erythrocyte membrane phospholipids of AP patients compared with healthy controls. Palmitic (C16:0), palmitoleic (C16:1n7cis), arachidonic (C20:4n6), docosahexaenoic (DHA, C22:6n3), and docosapentaenoic (DPA, C22:5n3) acids were the major contributing factors. A decrease in the peroxidation and unsaturation indexes in AP patients as well as the severe and mild AP groups as compared with controls was observed. The concentrations of antioxidant enzymes in the mild AP group were lower than in the control group. In severe AP of nonalcoholic etiology, the percentages of arachidic (C20:0) and arachidonic (C20:4n6) acids were decreased as compared with the control group. The patients with mild AP of nonalcoholic etiology had the increased percentages of total saturated FAs and gamma linoleic acid (C18:3n6) and the decreased percentages of elaidic (C18:1n9t), eicosapentaenoic acid (EPA, C20:5n3), DPA (C22:5n3), DHA (C22:6n3) as well as total and  $n-3$  PUFAs in erythrocyte membrane phospholipids.

**CONCLUSION:** The composition of FAs in erythrocyte membranes is altered during AP. These changes are likely to be associated with alcohol consumption, inflammatory processes, and oxidative stress.

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**Key words:** Acute pancreatitis; Alcohol; Fatty acids; Oxidative stress; Systemic inflammatory response syndrome

**Core tip:** The manuscript by Kuliaviene *et al.* elucidates the changes of fatty acids in erythrocyte membrane phospholipids during acute pancreatitis. Alcohol may influence the increased percentage of saturated and monounsaturated fatty acids of erythrocyte membrane. Fatty acids that are linked with inflammatory processes change differently during severe and mild nonalcoholic acute pancreatitis. The decrease of pro-inflammatory acids is seen in severe acute pancreatitis while anti-inflammatory players decrease during mild acute pancreatitis. The antioxidant enzymes of erythrocytes change in mild but not severe pancreatitis group. Thus the erythrocyte membranes can reflect the inflammatory and oxidative processes of acute pancreatitis.

Kuliaviene I, Gulbinas A, Cremers J, Pundzius J, Kupcinskas L, Dambrauskas Z, Jansen E. Fatty acids of erythrocyte membrane in acute pancreatitis patients. *World J Gastroenterol* 2013; 19(34): 5678-5684 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5678.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5678>

## INTRODUCTION

Acute pancreatitis (AP) is a sudden inflammation of pancreas. About 20%-30% of patients develop severe forms of the disease manifesting with local and systemic complications. Acute pancreatitis carries an overall mortality rate of 10%-15%<sup>[1,2]</sup>. The main causes of death are associated with multiple organ failure and pancreatic infection<sup>[3,4]</sup>. The initial process of inflammation starts in the pancreas, but no strict correlation between pancreatic necrosis and organ failure has been reported<sup>[5]</sup>. Systemic inflammatory response is responsible for multiple organ failure and has the most considerable impact on the severity of acute pancreatitis and mortality from this disease<sup>[6]</sup>.

The role of fatty acids (FAs) in the pathogenesis of AP is important but far from being clear. An increase the total serum free FA level is observed during AP<sup>[7]</sup>. Unsaturated FAs, especially polyunsaturated FAs (PUFAs), are liberated from pancreatic necrotic tissues and are responsible for the disturbance of FA profile in the serum of patients with AP<sup>[7,8]</sup>. The increased amount of unsaturated FAs in the necrotic pancreatic tissue and serum during AP is associated with multisystem organ failure and worse outcomes of patients<sup>[9]</sup>. Moreover, alcohol, an important etiological factor for pancreatitis, has an impact on the FA composition of serum and erythrocyte membranes<sup>[10-13]</sup>. Surprisingly, during alcohol-induced pancreatitis, the percentage of PUFAs is decreased in the serum FA profile in mild and moderate AP as well as chronic pancreatitis<sup>[14,15]</sup>. These data suggest that alcohol

could play a specific role in the pathogenesis of pancreatitis.

FAs of cell membranes are precursors for lipid mediators and play an important role in the process of inflammation and oxidant status<sup>[16]</sup>. Experimental findings show that n-3 PUFAs may be beneficial in the prevention of oxidative stress-induced inflammation in pancreatitis<sup>[17]</sup>. Moreover, it influences the histological severity of AP<sup>[18-21]</sup>. Human studies also indicate likely clinical benefits of enteral feeding rich in n-3 PUFAs in patients with AP<sup>[22]</sup>.

The aim of our study was to evaluate changes in the FA profile of erythrocyte membrane phospholipids and antioxidant enzymes of erythrocytes in patients with severe and mild AP, also of nonalcoholic etiology separately, in comparison with healthy individuals. We believe that erythrocyte membrane phospholipids can better reflect systemic changes caused by oxidative stress and inflammatory response in patients with AP comparing with FAs in serum, which are greatly influenced by necrotic changes in the pancreas and peripancreatic tissues. To our knowledge, no studies examining the FA composition of erythrocyte membrane phospholipids during AP have been carried out.

## MATERIALS AND METHODS

### Patients

All consecutive patients with a diagnosis of AP and onset of the disease within the last 72 h admitted to the Departments of Surgery and Gastroenterology at the Hospital of Lithuanian University of Health Sciences between June and December 2007 were included in this study. The diagnosis was established based on acute abdominal pain, at least 3-fold elevated levels of serum amylase, and typical radiological findings. According to the Acute Physiology and Chronic Health Evaluation (APACHE II) scale, the patients were subdivided into the mild (APACHE II score < 7,  $n = 22$ ) and severe (APACHE II score  $\geq 7$ ,  $n = 17$ ) AP groups. Healthy subjects ( $n = 26$ ) without a past history of pancreatic diseases were enrolled as controls.

### Fatty acid and antioxidant analysis

Peripheral blood samples were drawn from patients on admission to the hospital. Plasma and leukocytes were removed after centrifugation. Erythrocytes were washed and centrifuged twice. The samples were stored at -80 °C until analysis. The blood samples of the control group were subjected to the same procedure.

FA analysis was performed in the Laboratory for Health Protection Research, National Institute for Public Health and the Environment (The Netherlands), as described previously<sup>[23]</sup>. Briefly, 200  $\mu$ L of erythrocytes was taken, and phospholipids were washed with distilled water and extracted with chloroform/methanol (1:1). The chloroform layer was evaporated, and the phospholipids were hydrolyzed and methylated simultaneously

**Table 1** Demographic and clinical data of patients and controls

	AP	Severe AP	Mild AP	Control
Age, mean ± SD, yr	48.1 ± 15.5	50.2 ± 13.7	46.7 ± 16.8	42.07 ± 16.6
Men	68%	64%	70%	33%
AP etiology				
Alcoholic	35%	36%	35%	NA
Nonalcoholic	65%	64%	65%	NA
Death	6%	7%	5%	NA

Values are percentage unless otherwise stated. NA: Not applicable; AP: Acute pancreatitis.

with BF<sub>3</sub>/MeOH for 60 min at 100 °C. After extraction with hexane, the methylated FAs (FAME) were separated on a fused silica capillary column using a GC-3900 gas chromatograph with FID detection (Varian Assoc). The baseline separation of more than 50 FAME peaks was accomplished using FAME standards (Sigma) within 57 min. Individual FAs were expressed as percentages of the total FAs present in the chromatogram.

The concentrations of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured in erythrocytes on an auto analyzer (LX-20 Pro, Beckman-Coulter, Woerden, Netherlands) with kits from Randox (Ransod and Ransel, Crumlin, United Kingdom).

### Peroxidation and unsaturation index

The indexes were calculated according to the formulas used by Viviani *et al.*<sup>24</sup>. The peroxidation index (PI) was determined from the percentages of monoenoic, dienoic, trienoic, tetraenoic, pentaenoic and hexanoic FAs according to the following formula: PI = [(%monoenoic × 0.025) + (%dienoic × 1) + (%trienoic × 2) + (%tetraenoic × 4) + (%pentaenoic × 6) + (%hexanoic × 8)].

The unsaturation index (UI) is also known as the index of hydrogen deficiency. It was calculated from the number of unsaturated double bonds of each FA: UI = [(%monoenoic × 1) + (%dienoic × 2) + (%trienoic × 3) + (%tetraenoic × 4) + (%pentaenoic × 5) + (%hexanoic × 6)].

### Ethics

The study was approved by Kaunas Regional Ethics Committee for Biomedical Research (BE-2-47). All patients and healthy subjects provided written informed consent.

### Statistical analysis

Statistical analysis was performed using SPSS® for Windows release 14.0 (SPSS, Chicago, IL, United States). The data are presented as mean ± SD. The Mann-Whitney test and one-way and two-way ANOVA tests were applied for analysis of variables. All statistical tests were two sided, and *P* < 0.05 was considered statistically significant.

## RESULTS

The demographic characteristics of patients and controls

**Table 2** Percentages of saturated, monounsaturated and polyunsaturated fatty acids in erythrocyte membrane phospholipids

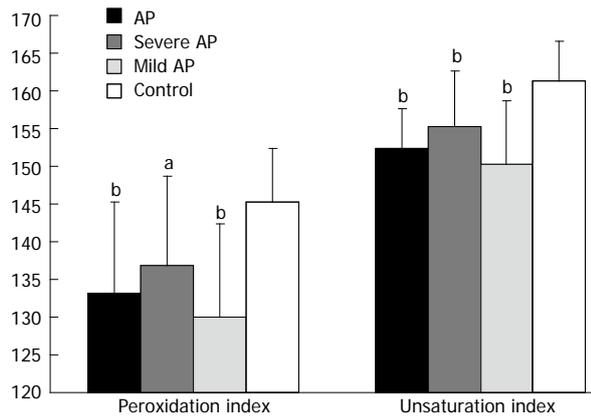
	AP	Alcoholic AP	Nonalcoholic AP	Control
Saturated fatty acid				
C14:0	0.26 ± 0.06	0.26 ± 0.06	0.26 ± 0.07	0.27 ± 0.06
C15:0	0.29 ± 0.05	0.27 ± 0.04	0.30 ± 0.06	0.30 ± 0.05
C16:0	23.43 ± 1.12 <sup>b</sup>	23.80 ± 1.20 <sup>b</sup>	23.22 ± 1.06 <sup>b</sup>	22.17 ± 0.85
C17:0	0.27 ± 0.06 <sup>b</sup>	0.23 ± 0.03 <sup>b</sup>	0.29 ± 0.06	0.31 ± 0.03
C18:0	13.49 ± 0.70 <sup>a</sup>	13.40 ± 0.54	13.54 ± 0.78	13.95 ± 0.70
C20:0	0.33 ± 0.10 <sup>b</sup>	0.28 ± 0.06 <sup>b</sup>	0.36 ± 0.09	0.40 ± 0.06
C21:0	0.04 ± 0.04	0.04 ± 0.06	0.04 ± 0.04	0.03 ± 0.03
C22:0	1.39 ± 0.33	1.15 ± 0.23 <sup>b</sup>	1.47 ± 0.34	1.44 ± 0.26
C23:0	0.21 ± 0.06 <sup>a</sup>	0.17 ± 0.04 <sup>b</sup>	0.23 ± 0.06	0.24 ± 0.04
C24:0	4.44 ± 0.80	4.30 ± 0.77	4.41 ± 0.97	4.10 ± 0.73
Total	44.05 ± 1.47 <sup>a</sup>	43.90 ± 1.00	44.13 ± 1.70	43.34 ± 0.90
Monounsaturated fatty acid				
C16:1n7 <i>trans</i>	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
C16:1n9c	0.09 ± 0.06 <sup>b</sup>	0.09 ± 0.03	0.09 ± 0.07	0.06 ± 0.03
C16:1n7c	0.56 ± 0.19 <sup>b</sup>	0.68 ± 0.17 <sup>b</sup>	0.49 ± 0.17 <sup>b</sup>	0.33 ± 0.06
C18:1n9 <i>trans</i>	0.11 ± 0.05 <sup>b</sup>	0.10 ± 0.02 <sup>b</sup>	0.12 ± 0.06 <sup>a</sup>	0.16 ± 0.04
C18:1n7 <i>trans</i>	0.28 ± 0.09 <sup>a</sup>	0.23 ± 0.07 <sup>b</sup>	0.30 ± 0.09	0.33 ± 0.09
C18:1n9c	13.02 ± 1.49	13.53 ± 1.05 <sup>a</sup>	12.85 ± 1.88	12.42 ± 1.12
C18:1n7c	1.08 ± 0.17	1.09 ± 0.15	1.07 ± 0.20	1.03 ± 0.13
C22:1n9c	0.22 ± 0.05 <sup>a</sup>	0.23 ± 0.06 <sup>a</sup>	0.22 ± 0.05 <sup>a</sup>	0.18 ± 0.05
C24:1	4.98 ± 0.69	4.86 ± 0.63	4.87 ± 0.86	4.58 ± 0.67
Total	20.49 ± 1.98 <sup>a</sup>	20.93 ± 1.13 <sup>a</sup>	20.16 ± 2.29	19.31 ± 1.02
PUFA				
Omega 3 PUFA	7.84 ± 1.71 <sup>b</sup>	7.92 ± 1.74 <sup>a</sup>	7.80 ± 1.74 <sup>b</sup>	9.45 ± 1.30
C18:3n3	0.16 ± 0.05	0.18 ± 0.06	0.14 ± 0.04	0.15 ± 0.03
C20:5n3	0.91 ± 0.46	0.94 ± 0.46	0.82 ± 0.42	1.02 ± 0.4
C22:5n3	2.21 ± 0.33 <sup>a</sup>	2.29 ± 0.36	2.17 ± 0.32 <sup>a</sup>	2.39 ± 0.29
C22:6n3	4.61 ± 1.12 <sup>b</sup>	4.50 ± 1.12 <sup>b</sup>	4.67 ± 1.14 <sup>b</sup>	5.89 ± 0.81
Omega 6 PUFA	26.99 ± 1.99	26.89 ± 1.40	27.51 ± 2.17	27.93 ± 1.58
C18:2n6c	10.16 ± 1.24	10.07 ± 1.01	10.43 ± 1.43	10.18 ± 1.27
C18:3n6	0.05 ± 0.02 <sup>b</sup>	0.05 ± 0.02 <sup>b</sup>	0.04 ± 0.02 <sup>a</sup>	0.03 ± 0.01
C20:3n6	1.44 ± 0.26 <sup>a</sup>	1.49 ± 0.27	1.41 ± 0.25	1.32 ± 0.34
C20:4n6	12.94 ± 1.06 <sup>a</sup>	12.82 ± 0.82 <sup>a</sup>	13.01 ± 1.18 <sup>a</sup>	13.64 ± 0.99
C22:4n6	2.48 ± 0.58	2.46 ± 0.35	2.62 ± 0.60	2.63 ± 0.50
C16:3n4	0.05 ± 0.02 <sup>a</sup>	0.04 ± 0.02 <sup>b</sup>	0.06 ± 0.02	0.06 ± 0.02
C20:2	0.28 ± 0.06 <sup>a</sup>	0.27 ± 0.05	0.29 ± 0.06 <sup>a</sup>	0.25 ± 0.03
C22:2	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.02
Total PUFA	35.51 ± 1.95 <sup>b</sup>	35.17 ± 1.37 <sup>b</sup>	35.71 ± 2.21 <sup>b</sup>	37.34 ± 1.30

Results are presented as mean ± SD. Percentages of saturated, monounsaturated and polyunsaturated fatty acids (PUFA) in erythrocyte membrane phospholipids in patients with acute pancreatitis (AP) of alcoholic and nonalcoholic etiology. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group.

are presented in Table 1. There was no difference in the FA composition of membrane phospholipids between men and women in the control group (data not shown).

As shown in Table 2, the percentage of saturated FAs in erythrocyte membrane phospholipids was greater in the patients with AP than the control group. Palmitic acid (C16:0) had a major impact on the increase. The percentages of monounsaturated FAs were also increased, and *cis*-isomers, especially palmitoleic (C16:1n7*cis*) and erucic (C22:1n9*cis*) acids, mostly contributed to the change. We found a decreased percentage of some *trans* monounsaturated FAs in erythrocyte membrane phospholipids. Those were elaidic (C8:1n9*trans*) and vaccenic (C18:1n7*trans*) acids (Table 2).

The percentages of total and n-3 PUFAs were decreased in erythrocyte membrane phospholipids of AP



**Figure 1** Peroxidation and unsaturation indexes of fatty acids of erythrocyte membrane phospholipids in the acute pancreatitis, severe acute pancreatitis, mild acute pancreatitis, and control groups. A significant decrease of peroxidation index and unsaturation index in acute pancreatitis (AP) patients compared with controls was observed. Bars represent mean values with standard deviation. The extent of change in the indexes in mild AP was greater than in severe AP patients comparing with controls (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control group).

patients. This was particularly caused by the decreased percentages of docosahexaenoic (DHA C22:6n3) and docosapentaenoic (DPA C22:5n3) acids. The percentage of arachidonic acid (AA, C20:4n6) was decreased in the patients with AP comparing with the controls, though the percentages of gamma-linoleic (C18:3n6) and dihomo gamma linoleic (C20:3n6) acids were increased (Table 2).

A decrease of PI and UI in AP patients as well as the severe and mild AP groups compared with the controls was observed. The extent of change in the indexes in the mild AP group was greater than in the severe AP group compared with controls (Figure 1). The concentrations of SOD and GPx in the mild AP group were lower comparing with the control group ( $321.55 \pm 75.19 \mu\text{mol}/\text{mL}$  and  $10059.21 \pm 2666.96 \text{ U}/\text{L}$  vs  $384.88 \pm 42.21 \mu\text{mol}/\text{mL}$  and  $11649.09 \pm 1844.75 \text{ U}/\text{L}$ ,  $P < 0.001$  and  $P < 0.05$ , respectively). To rule out the impact of alcohol in the changes of FAs in erythrocyte membrane phospholipids of AP patients, we analyzed AP of non-alcoholic etiology (Table 3). In the severe AP group, the percentages of arachidic acid (C20:0) and AA (C20:4n6) were decreased as compared with the control group. In the mild AP group, an increase in the percentages of total saturated FAs and gamma linoleic acid (C18:3n6) and a decrease in the percentages of elaidic (C18:1n9t), EPA (C20:5n3), DPA (C22:5n3), DHA (C22:6n3), total PUFAs, and omega 3 PUFAs in erythrocyte membrane phospholipids were recorded. The change in the concentrations of antioxidant enzymes showed the same pattern: they were decreased in patients with mild AP and showed no change in the severe AP group. The changes in the PI and the UI appeared to be greater in the mild AP than severe AP group as compared with the control group.

## DISCUSSION

This study has analyzed the impact of systemic inflam-

matory response and oxidative stress on the FA composition of erythrocyte membranes and the concentrations of enzymes in patients with AP. The initial generation of ROS and inflammatory events occur in the pancreas, but systemic changes have a crucial impact on the severity and fatal outcomes of AP<sup>[6]</sup>. A better understanding of these systemic processes occurring during AP could help identifying new therapeutic treatment options and escaping undesirable complications or fatal outcomes.

In our study, we found that the FA composition of erythrocyte membrane phospholipids was significantly altered during AP compared with controls mainly because of the increased percentages of saturated and monounsaturated acids, namely palmitic and palmitoleic, and a decreased percentage of PUFAs. Contrary, Sztéfko *et al*<sup>[7]</sup> found that the proportion of saturated and monounsaturated acids was decreased and the proportion of PUFAs was increased in the serum levels of free FAs in patients with AP. An increase in the percentage of PUFAs in the necrotic pancreatic tissue has also been reported<sup>[8]</sup>. On the other hand, in severe sepsis, a similar pathology with systemic inflammatory response syndrome, the lower proportions of PUFAs and the greater proportions of monounsaturated FAs in erythrocyte phospholipids have been documented<sup>[25]</sup>. These findings suggest that the FA composition of erythrocyte membrane phospholipids may reflect not only the direct events in the pancreas, but also the systemic response syndrome during AP.

Alcohol consumption can be associated with the higher percentages of saturated and monounsaturated FAs, such as palmitic and oleic acids, and the lower percentages of PUFAs, especially DHAs and arachidonic acid, in serum and membranes<sup>[10-13]</sup>. Alcoholics have also been shown to have a disturbed oxidant status of plasma and erythrocyte enzymes<sup>[26-28]</sup>. In the study by Khan *et al*<sup>[14]</sup>, the authors showed the increased percentages of saturated palmitic and monounsaturated FAs as well as the decreased percentages of some PUFAs in serum of patients with alcohol-induced AP comparing with alcoholic controls. Moreover, Gabianelli *et al*<sup>[29]</sup> reported that ethanol can have a direct toxic effect on erythrocyte membranes and antioxidant systems of the cells. These findings indicate that alcohol may have an impact on the FA composition of erythrocyte membrane phospholipids. Thus, the increased percentages of saturated and monounsaturated FAs in our study could partly be explained by etiological factors, most probably alcohol.

To rule out the impact of alcohol and to study the influence of inflammatory and oxidative processes during AP on the phospholipid composition of erythrocyte membranes, we analyzed patients with AP of nonalcoholic etiology. The PUFAs of cell membranes are precursors for prostaglandins and other lipid mediators of inflammatory process<sup>[16]</sup>. Arachidonic acid is the main proinflammatory actor. Meanwhile, EPA, DHA, and possibly DPA are precursors for products with anti-inflammatory and proresolving functions<sup>[30,31]</sup>. We found that in the severe AP group, the percentage of proinflammatory arachidonic acid was significantly decreased, and

**Table 3 Percentages of fatty acids**

	Nonalcoholic		Control
	Severe AP	Mild AP	
C20:0	0.32 ± 0.09 <sup>d</sup>	0.39 ± 0.09	0.40 ± 0.06
C20:4n6	12.89 ± 0.83 <sup>c</sup>	13.09 ± 1.40	13.64 ± 0.99
SFA	43.53 ± 1.29	44.54 ± 1.86 <sup>c</sup>	43.34 ± 0.90
C18:3n6	0.04 ± 0.02	0.05 ± 0.02 <sup>c</sup>	0.03 ± 0.01
C18:1n9t	0.13 ± 0.04	0.12 ± 0.07 <sup>d</sup>	0.16 ± 0.04
C20:5n3	1.03 ± 0.56	0.67 ± 0.18 <sup>d</sup>	1.02 ± 0.4
C22:5n3	2.28 ± 0.22	2.10 ± 0.36 <sup>c</sup>	2.39 ± 0.29
C22:6n3	5.29 ± 1.02	4.24 ± 1.05 <sup>d</sup>	5.89 ± 0.81
n-3 PUFA	8.76 ± 1.73 <sup>a</sup>	7.14 ± 1.45 <sup>d</sup>	9.45 ± 1.30
Total PUFA	36.87 ± 1.62 <sup>a</sup>	34.91 ± 2.26 <sup>d</sup>	37.34 ± 1.30
SOD	385.37 ± 21.37 <sup>a</sup>	314.90 ± 86.04 <sup>d</sup>	384.88 ± 42.21
GPx	12557.07 ± 2836.06 <sup>a</sup>	9370.85 ± 2196.13 <sup>d</sup>	11649.09 ± 1844.75
PI	138.49 ± 10.34 <sup>a,c</sup>	128.14 ± 10.74 <sup>d</sup>	146.19 ± 7.08
UI	156.67 ± 7.28 <sup>a,c</sup>	148.99 ± 8.04 <sup>d</sup>	161.70 ± 4.90

Results are presented as mean ± SD. Percentages of fatty acids in erythrocyte membrane phospholipids, peroxidation and unsaturation indexes, and concentrations of superoxide dismutase (SOD,  $\mu\text{mol}/\text{mL}$ ) and glutathione peroxidase (GPx, U/L) in patients with acute pancreatitis (AP) of nonalcoholic etiology. <sup>a</sup> $P < 0.05$  comparing mild and severe AP; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs control group. PUFA: Polyunsaturated fatty acids; SFA: Saturated fatty acids.

in the mild AP group, a decrease in the percentages of anti-inflammatory players (EPA, DHA, and DPA) was seen as compared with controls. It is now thought that saturated FAs could also be involved in the inflammatory process<sup>[32,33]</sup>. We also found a significant increase in the percentage of total saturated FAs in the mild but not severe AP group. Erythrocytes are not usually considered to be active players in the inflammatory process, but our study showed that the changes in the percentage of FAs in erythrocyte membrane phospholipids were different during mild and severe AP, therefore, we hypothesize that the composition of erythrocyte membrane phospholipids may reflect the inflammatory processes and the severity of the disease.

Oxidative stress plays a central role in the development of pancreatic inflammation and extra pancreatic complications<sup>[34-36]</sup>. The changes of the FA composition of erythrocyte membrane phospholipids could be affected from “the outside” as PUFAs of erythrocyte membrane phospholipids are extremely sensitive to oxidation<sup>[37]</sup>. SOD and GPx are important components of enzymatic antioxidant defense<sup>[37]</sup>. We found that the levels of antioxidant enzymes in erythrocytes were also altered significantly in the mild but not severe AP group comparing with controls. There were significant differences in the PI and the UI mainly because of the different percentage of PUFAs in erythrocyte membrane phospholipids in the severe and mild AP groups comparing with controls. Moreover, there was a significant difference between the severe and mild AP groups. This suggests that oxidative stress might be involved in the changes of the FA composition of erythrocyte membrane phospholipids and systemic inflammatory response.

It was unexpected to find the changes of PI and UI to be more apparent in the mild AP than severe AP group comparing with controls. It is known that systemic response followed by organ failure influences the severity

and outcome of AP more than the events in the pancreas itself<sup>[5]</sup>. The similar phenomenon was also noticed in cytokine expression during mild and severe AP<sup>[38]</sup>. We hypothesize that these findings could reflect the disproportion of pro- and anti-inflammatory processes during severe AP possibly associated with oxidative stress. The mechanisms of these processes are still not clear and remain to be elucidated.

The composition of FAs in erythrocyte membranes is altered during AP. These changes are likely to be associated with alcohol intake as an etiological factor for AP, and systemic inflammatory processes and oxidative stress after the onset of the disease could influence the changes.

## COMMENTS

### Background

Acute pancreatitis carries an overall mortality rate of 10%-15%. Systemic inflammatory response has the most considerable impact on the severity of acute pancreatitis and mortality from this disease. Fatty acids of cell membranes are precursors for lipid mediators and play an important role in the process of inflammation and oxidant status. Erythrocyte membrane phospholipids can reflect systemic changes caused by oxidative stress and inflammatory response in patients with acute pancreatitis.

### Research frontiers

The role of fatty acids in the pathogenesis of acute pancreatitis is important but far from being clear. Fatty acids are the components of membrane phospholipids. They are responsible for inflammatory and oxidative processes. Free fatty acids in serum are associated with necrotic lesions in the pancreas and peripancreatic tissues. Moreover, alcohol, an important etiological factor for pancreatitis, has an impact on the fatty acid composition of serum and erythrocyte membranes.

### Innovations and breakthroughs

The earlier studies of alterations in the fatty acid composition during acute pancreatitis mainly investigated the fatty acid composition in serum that is greatly influenced by necrotic changes in the pancreas and peripancreatic tissues. Authors believe that erythrocyte membrane phospholipids can better reflect systemic changes caused by oxidative stress and inflammatory response as well as alcohol impact in patients with acute pancreatitis. To the knowledge, no studies examining the fatty acid composition of membranes during acute pan-

creatitis have been carried out.

### Applications

This study elucidates the pathogenesis of acute pancreatitis, especially the systemic and oxidative processes that are of high importance in the severity of acute pancreatitis and mortality from this disease.

### Terminology

Fatty acids are the components of phospholipids that form the lipid bilayers of cell membranes. Fatty acids of membranes are precursors for lipid mediators of inflammatory response syndrome. Oxidative stress is a disturbance of the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage, and is associated with many chronic and acute inflammatory conditions.

### Peer review

This study evaluates changes in fatty acids of erythrocyte membrane phospholipids during mild and severe acute pancreatitis, of alcohol and non-alcohol etiology. The study is a prospective one and Acute Physiology and Chronic Health Evaluation II score was used to classify patients into mild ( $n = 22$  patients) and severe ( $n = 17$  patients). Some 26 healthy individuals were enrolled as control. This is a well conducted prospective study, and to my knowledge this is the first study that examines the fatty acid profile of the erythrocyte membrane in acute pancreatitis.

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## Diversity of *Helicobacter pylori* genotypes in Iranian patients with different gastroduodenal disorders

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### Abstract

**AIM:** To investigate the diversity of *Helicobacter pylori* (*H. pylori*) genotypes and correlations with disease outcomes in an Iranian population with different gastroduodenal disorders.

**METHODS:** Isolates of *H. pylori* from patients with different gastroduodenal disorders were analyzed after culture and identification by phenotypic and genotypic methods. Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Germany). After DNA extraction, genotyping was done for *cagA*, *vacA* (s and m regions), *iceA* (*iceA*<sub>1</sub>, *iceA*<sub>2</sub>) and *babA* with specific primers for each allele using polymerase chain reaction (PCR). All patients' pathologic and clinical data and their relation with known genotypes were analyzed by using SPSS version 19.0 software.  $\chi^2$  test and Fisher's exact test were used to assess relationships between categorical variables. The level of statistical significance was set at  $P < 0.05$ .

**RESULTS:** A total of 71 isolates from 177 patients with different gastroduodenal disorders were obtained. Based on analysis of the *cagA* gene (positive or negative), *vacA* s-region (*s*<sub>1</sub> or *s*<sub>2</sub>), *vacA* m-region (*m*<sub>1</sub> or *m*<sub>2</sub>), *iceA* allelic type (*iceA*<sub>1</sub> and *iceA*<sub>2</sub>) and *babA* gene (positive or negative), twenty different genotypic combinations were recognized. The prevalence of *cagA*, *vacA* *s*<sub>1</sub>, *vacA* *s*<sub>2</sub>, *vacA* *m*<sub>1</sub>, *vacA* *m*<sub>2</sub>, *iceA*<sub>1</sub>, *iceA*<sub>2</sub>, *iceA*<sub>1</sub>+*iceA*<sub>2</sub> and *babA* were 62%, 78.9%, 19.7%, 21.1%, 78.9%, 15.5%, 22.5%, 40.8% and 95.8%, respectively. Interestingly, evaluation of PCR results for *cagA* in 6 patients showed simultaneous existence of *cagA* variants according to their size diversities that proposed mixed infection in these patients. The most prevalent genotype in *cagA*-positive isolates was *cagA*<sup>+</sup>/*vacA*<sub>s1</sub>/*m*<sub>2</sub>/*iceA*<sub>1</sub>+*A*<sub>2</sub>/*babA*<sup>+</sup> and in *cagA*-negative isolates was *cagA*<sup>-</sup>/*vacA*<sub>s1</sub>/*m*<sub>2</sub>/*iceA*<sub>1</sub>-/*babA*<sup>+</sup>. There were no relationships between the studied genes and histo-

pathological findings (*H. pylori* density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy). The strains which carry *cagA*, *vacAs1/m1*, *iceA2* and *babA* genes showed significant associations with severe active chronic gastritis ( $P = 0.011$ ,  $0.025$ ,  $0.020$  and  $0.031$ , respectively). The *vacAs1* genotype had significant correlation with the presence of the *cagA* gene ( $P = 0.013$ ). Also, *babA* genotype showed associations with *cagA* ( $P = 0.024$ ). In the combined genotypes, only *cagA*<sup>+</sup>/*vacAs1/m1/iceA2/babA*<sup>+</sup> genotype showed correlation with severe active chronic gastritis ( $P = 0.025$ ).

**CONCLUSION:** This genotyping panel can be a useful tool for detection of virulent *H. pylori* isolates and can provide valuable guidance for prediction of the clinical outcomes.

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**Key words:** *Helicobacter pylori*; *cagA*; *vacA*; *iceA*; *babA*

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## INTRODUCTION

Infection with *Helicobacter pylori* (*H. pylori*) causes different clinical disorders such as persistent gastritis, peptic ulcers and mucosa associated lymphoid tissue (MALT) lymphoma. Current studies suggest that *H. pylori* infection may be a crucial risk factor in the development of gastric cancer<sup>[1,2]</sup>. In this regard, this pathogen has been categorized as a group I carcinogen by the International Agency for Research on Cancer<sup>[3]</sup>. The detailed reasons for these different clinical outcomes are unknown, but they may be related to host genetic factors, exposure to environmental factors (*e.g.*, diet, drug usage, acidity of the stomach and smoking) and to the bacterial genotypes<sup>[4]</sup>. *H. pylori* shows extensive genetic diversity and this variability has a crucial role in pathogenesis of this bacterium<sup>[5]</sup>. Several *H. pylori* virulence factor genes related to the risk of gastroduodenal disorders, including *cagA*, *vacA*, *babA* and *iceA*, have been proposed<sup>[6]</sup>. A tremendous number of studies have proved that CagA and VacA producing strains are related to severe clinical outcomes<sup>[7]</sup>. In addition to *cagA* and *vacA*, the other *H. pylori* virulence factors, such as *iceA* and *babA*, also showed such associations in some studies<sup>[8,9]</sup>. Beyond the role of these factors in progression of the disease, there are several papers which reported a relationship between failure of *H. pylori* eradication therapy and the strains' virulence factor genotypes<sup>[10]</sup>. Analysis of genetic structure of virulence factors among the isolates from different geographic regions will provide new insights regarding the pathogenesis and treatment of *H.*

*pylori* infection. *H. pylori* genotyping may have multiple roles including impact on the cure rates of eradication therapy<sup>[10]</sup>, determination of clinical outcomes<sup>[11]</sup>, tracking human migration<sup>[12,13]</sup> and recently, the prediction of progression of gastric preneoplastic lesions<sup>[14]</sup>. The distribution pattern of *H. pylori* genotypes and its correlation with disease outcome shows geographic differences. The aim of this study was to assess the diversity of *H. pylori* genotypes in an Iranian population to determine genotypically the *H. pylori* isolates more associated with different gastroduodenal disorders.

## MATERIALS AND METHODS

### Clinical specimens

Three gastric biopsies (two were used for histological examination and one for culture) were obtained from 177 adult patients undergoing routine diagnostic endoscopy referred to the Endoscopy Centre of Taleghani Hospital of Tehran, Iran, after obtaining informed consent. All subjects answered questionnaires related to age, sex, gastric or duodenal peptic ulcer diseases upon endoscopy.

### Culture

Antral or body biopsy specimens from each patient were kept in transport medium consisting of thioglycolate with 1.3 g/L agar (Merck) and 3% yeast extract (Oxoid). The endoscopic biopsy specimens were cut into small pieces, homogenized with a sterile scalpel and were smeared on the surface of Brucella agar plates supplemented with 7% horse blood and Campylobacter selective supplement (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg) and amphotericin B (2.5 mg/L). Incubation was performed in microaerophilic conditions at 37 °C for 5-7 d. Identification of *H. pylori* isolates was performed by analyzing colony morphology, Gram staining, oxidase, catalase and urease activities and *H. pylori*-specific polymerase chain reaction (PCR) (*glmM*). The isolates were preserved in BHI broth containing 20% glycerol and 10% fetal calf serum and stored at -70 °C.

### DNA extraction

Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was stored at -20 °C until used for molecular studies.

### *H. pylori* genotyping

After DNA extraction, polymerase chain reactions (PCR) were performed in a volume of 25 µL containing 1 × PCR buffer, 1 µmol/L of each primer, 1 µL of genomic DNA (approximately 150 ng), 200 µmol/L of dNTPs mix, 2 mmol/L of MgCl<sub>2</sub>, and 0.05 U/µL Taq DNA polymerase. PCR amplifications were performed in an automated thermal cycler (AG 22331; Eppendorf, Hamburg, Germany) under the following conditions: for *vacA s/m*: 33 cycles of 1 min at 94 °C, 33 s at 55 °C, and 1 min at 72 °C; for *cagA*: 33 cycles of 1 min at 94 °C, 1 min at

Table 1 Primers used in this study

Gene	Primers (5'→3')	PCR product (bp)	Annealing temperature (°C)	Ref.
<i>vacA</i> (s <sub>1</sub> /s <sub>2</sub> )	VA1F: ATGGAAATACAACAAACACAc VA1R: CTGCTTGAATGCGCCAAAC	259-286	55	[6]
<i>vacA</i> (m <sub>1</sub> /m <sub>2</sub> )	VACm1m2F: CAATCTGTCCAATCAAGCGAG VACm1m2R: GCGTCAAAATAATTCCAAGG	567-642	55	[15]
<i>cagA</i>	CagAF: AATACACCAACGCCTCCAAG CagAR: TTGTTGCCGCTTTIGCTCTC	400	59	[16]
<i>iceA</i> <sub>1</sub>	iceA1F: TATTTCTGGAACCTTGCGCAACCTGAT M.Hpy1R: GGCCTACAACCGCATGGATAT	approximately 900	58	[17]
<i>iceA</i> <sub>2</sub>	iceA2 F: CGGCTGTAGGCACTAAAAGCTA iceA2 R: TCAATCCTATGTGAAACAATGATCGTT	approximately 800	58	[17]
<i>babA</i>	babAF: CCAAACGAAACAAAAAGCGT babAR: GCTTGTGTAAGCCGTCGT	271	58	[18]
<i>glmM</i>	GlmM2-F GGATAAGCTTTTAGGGGTGTAGGGG GlmM1-R GCTTACTTTCTAACACTAACCGCGC	296	52	[19]

59 °C, and 1 min at 72 °C; for *iceA*<sub>1</sub>/*A*<sub>2</sub>: 33 cycles of 1 min at 94 °C, 40 s at 58 °C, and 1 min at 72 °C, and for *babA*: 35 cycles of 1 min at 94 °C, 40 s at 58 °C and 1 min at 72 °C. The amplified genes were detected by electrophoresis in a 1.2% agarose gel with ethidium bromide. Table 1 summarizes the primer sequences, annealing temperatures and the expected size of the PCR products.

### Histopathological evaluation

Sections were stained with hematoxylin and eosin for analysis of *H. pylori*-related histology by an expert pathologist. Then the grade of gastritis was scored based on the updated Sydney System.

### Statistical analysis

Data were analyzed by using SPSS version 19.0.0 software (IBM, IL, United States).  $\chi^2$  test and Fisher's exact test were used to assess relationships between categorical variables. The level of statistical significance was set at  $P < 0.05$ .

## RESULTS

### Infection rates and clinical disorders

A total of 71 isolates from 177 patients (parenthesis approximately 40%) with different gastroduodenal disorders were obtained. The *H. pylori*-positive patients consisted of 24 males and 47 females, with their ages ranging between 19 and 85 years (mean age, 66 years). All of the isolates showed positive results for the common identification test and *H. pylori*-specific PCR (*glmM*). Most of the infected patients suffered from chronic gastritis (84.6%), while the others showed duodenitis (9.8%), intestinal metaplasia (2.8%), hyperplasia (1.4%) and gastric cancer diseases (1.4%) (Table 2).

### Allelic diversities in main putative virulence markers

***cagA* genotyping:** The 400-bp PCR product indicating the presence of the *cagA* gene was obtained in 44 isolates (62%) and 27 (38%) were negative. Interestingly, evaluation of PCR results for *cagA* in 6 patients showed simultaneous existence of *cagA* variants according to their size

diversities.

***vacA* genotyping:** The frequency of *vacA* s<sub>1</sub>, *vacA* s<sub>2</sub>, *vacA* m<sub>1</sub> and *vacA* m<sub>2</sub> were 78.9%, 19.7%, 21.1% and 78.9%, respectively. Only one isolate was *vacA* som2 (with no PCR product for s region).

***iceA* genotyping:** Sole existence of *iceA*<sub>1</sub> genotype was detected in 15.5% and *iceA*<sub>2</sub> genotype in 22.5% of the colonized patients. Interestingly, out of the total studied samples, 40.8% were infected with both *iceA*<sub>1</sub> and *iceA*<sub>2</sub> genotypes and 21.1% were negative for these genes.

***babA* genotyping:** *babA* was found in 68 of the patients (95.8%); however, three patients (4.2%) did not show this allelic variant (Figure 1).

### Correlation of *H. pylori* genotypes with pathological data, patients' age and clinical outcome

**Combination of genotypes:** Based on the analysis of the *cagA* gene (positive or negative), *vacA* s-region (s<sub>1</sub> or s<sub>2</sub>), *vacA* m-region (m<sub>1</sub> or m<sub>2</sub>), *iceA* allelic types (*iceA*<sub>1</sub> and *iceA*<sub>2</sub>) and *babA* (positive or negative), twenty different genotypic combinations were recognized. The most prevalent genotype in *cagA* positive isolates was *cagA*<sup>+</sup>/*vacA*s<sub>1</sub>m<sub>2</sub>/*iceA*<sub>1</sub>+*A*<sub>2</sub>+/*babA*+ and in *cagA* negative isolates was *cagA*<sup>-</sup>/*vacA*s<sub>1</sub>m<sub>2</sub>/*iceA*-/*babA*+ (Figure 2).

***Helicobacter pylori* density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy:** There was no significant relationship between *cagA* positivity and *H. pylori* density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy in the biopsies. Also no relationships were found between other genes and these histopathological findings.

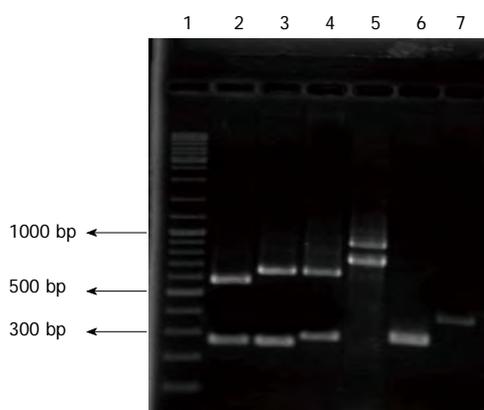
**Patients' age:** There was no significant relationship between the genotypes, clinical and pathological data and patients' age.

**Chronic gastritis:** The gastritis was scored as severe ac-

**Table 2 Association of combined genotypes with pathological conditions in *Helicobacter pylori* isolates**

Combination of genotypes	SCG	SACG <sup>2</sup>	MACG	MiACG	MCG	H	M	GC	D	Total	P value <sup>1</sup>
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA1+iceA2</i> / <i>babA</i> <sup>+</sup>	1	12	2	0	0	0	1	0	1	17	0.025 <sup>2</sup>
<i>cagA</i> <sup>+</sup> / <i>vacAs1m1</i> / <i>iceA2</i> / <i>babA</i> <sup>+</sup>	0	3	0	0	0	0	0	0	1	4	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA1</i> / <i>babA</i> <sup>+</sup>	0	3	1	0	0	0	1	0	2	7	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m1</i> / <i>iceA1+iceA2</i> / <i>babA</i> <sup>+</sup>	0	6	1	0	1	0	0	0	0	8	
<i>cagA</i> <sup>+</sup> / <i>vacAs2m2</i> / <i>iceA1+iceA2</i> / <i>babA</i> <sup>+</sup>	0	1	1	0	0	0	0	0	0	2	
<i>cagA</i> <sup>+</sup> / <i>vacAs0m2</i> / <i>iceA2</i> / <i>babA</i> <sup>+</sup>	0	0	0	0	0	0	0	0	1	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA2</i> / <i>babA</i> <sup>+</sup>	0	1	0	0	0	0	0	1	0	2	
<i>cagA</i> <sup>+</sup> / <i>vacAs2m2</i> / <i>iceA1</i> / <i>babA</i> <sup>+</sup>	0	0	1	0	0	0	0	0	0	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs2m2</i> / <i>iceA-</i> / <i>babA</i> <sup>+</sup>	0	1	0	0	0	0	0	0	0	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA-</i> / <i>babA</i> <sup>+</sup>	0	1	0	0	0	0	0	0	0	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA-</i> / <i>babA</i> <sup>+</sup>	0	3	2	0	1	0	0	0	1	7	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m1</i> / <i>iceA-</i> / <i>babA</i> <sup>+</sup>	0	1	0	0	1	0	0	0	0	2	
<i>cagA</i> <sup>+</sup> / <i>vacAs2m2</i> / <i>iceA2</i> / <i>babA</i> <sup>+</sup>	1	0	0	0	2	0	0	0	0	3	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA2</i> / <i>babA</i> <sup>+</sup>	0	2	2	0	0	1	0	0	0	5	
<i>cagA</i> <sup>+</sup> / <i>vacAs2m2</i> / <i>iceA1</i> / <i>babA</i> <sup>+</sup>	0	1	0	0	0	0	0	0	0	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m1</i> / <i>iceA2</i> / <i>babA</i> <sup>+</sup>	0	0	1	0	0	0	0	0	0	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs2m2</i> / <i>iceA-</i> / <i>babA</i> <sup>+</sup>	0	3	1	0	0	0	0	0	0	4	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA1+iceA2</i> / <i>babA</i> <sup>+</sup>	0	1	0	0	0	0	0	0	0	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs1m2</i> / <i>iceA1+iceA2</i> / <i>babA</i> <sup>-</sup>	0	0	0	1	0	0	0	0	0	1	
<i>cagA</i> <sup>+</sup> / <i>vacAs2m2</i> / <i>iceA1</i> / <i>babA</i> <sup>-</sup>	0	0	1	0	0	0	0	0	1	2	
Total	2	39	13	1	5	1	2	1	7	71	

<sup>1</sup>Only  $P < 0.05$  are indicated; <sup>2</sup>This  $P$  value is related to severe active chronic gastritis (SACG). SCG: Severe chronic gastritis; MACG: Moderate active chronic gastritis; MiACG: Mild active chronic gastritis; MCG: Moderate chronic gastritis; H: Hyperplasia; M: Metaplasia; GC: Gastric cancer; D: Duodenitis.



**Figure 1** Polymerase chain reaction products of the main putative virulence markers. Lane 1: DNA ladder mix; Lane 2: *vacAs1m1* genotype; Lane 3: *vacAs1m2* genotype; Lane 4: *vacAs2m2* genotype; Lane 5: *iceA1+iceA2* genotype; Lane 6: *babA* genotype; Lane 7: *cagA* genotype

tive chronic gastritis, moderate active chronic gastritis, mild active chronic gastritis, severe chronic gastritis and moderate chronic gastritis. The strains which carried the *cagA* gene showed significant associations with severe active chronic gastritis ( $P = 0.011$ ). Also, the strains which carried the *vacA* *s1/m1* gene showed significant associations with severe active chronic gastritis ( $P = 0.025$ ). *babA* ( $P = 0.031$ ) and *iceA2* ( $P = 0.020$ ) also had significant correlation with severe active chronic gastritis. In the combined genotypes this association was observed for *cagA*<sup>+</sup>/*vacAs1m1*/*iceA2*/*babA*<sup>+</sup> genotype in the case of severe active chronic gastritis ( $P = 0.025$ ).

**Genotype correlation:** Interestingly, the *vacA* *s1* geno-

type had significant correlation with the presence of the *cagA* gene ( $P = 0.013$ ). Also *babA* genotype showed this association in *cagA* positive isolates ( $P = 0.024$ ).

## DISCUSSION

*H. pylori* infection is usually present in 60%-80% of gastric and 95% of duodenal ulcers. However, some conditions affect infection rate of this bacterium in different geographic and socioeconomic regions. The prevalence of infection is typically higher in developing countries (greater than 80%) and lower in the developed ones (typically less than 40%)<sup>[20]</sup>. It has been demonstrated that the prevalence of *H. pylori* infection in developing countries with low socioeconomic status and poor management of drinking water is much higher (> 80%) than that in developed countries (< 60%)<sup>[21]</sup>. In our study the recovery rate of *H. pylori* was 40% which shows the improvement in the living conditions and hygiene in Iran that has also been reported recently<sup>[22]</sup>.

*H. pylori* can be divided into *cagA*-positive and *cagA*-negative strains, and there is increasing evidence that infection with *cagA*-positive isolates is associated with a greater risk of adverse clinical outcomes than infections with strains lacking this gene. In the current study, the strains which carried the *cagA* gene showed significant associations with severe active chronic gastritis. Interestingly, the prevalence of the *cagA*-positive strain differs among different countries, and more than 90% of *H. pylori* strains are *cagA*-positive in East Asian countries, irrespective of clinical presentation<sup>[23]</sup>. Sasaki *et al*<sup>[24]</sup> showed that among *H. pylori* DNA-positive samples, *cagA* was detected in 45.9% from Ecuador and 20.0% from Panama.

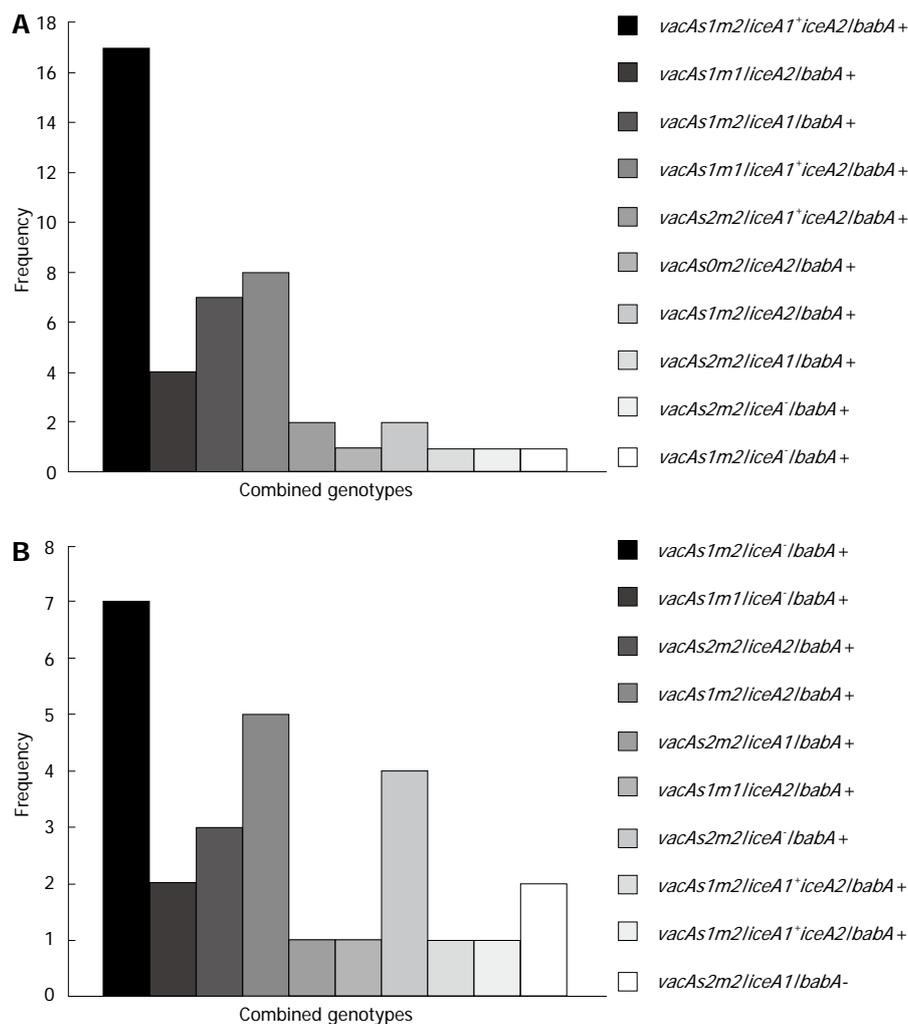


Figure 2 The frequency of combined genotypes. A: Combined *vacA*, *iceA* and *babA* genotypes in 44 *cagA* positive isolate; B: Combined *vacA*, *iceA* and *babA* genotypes in 27 *cagA* negative isolates.

In our study the prevalence of *cagA*-positive isolates is 62% which is less than other Asian countries and more than other countries (e.g., Ecuador, Panama). According to Watada *et al.*<sup>[25]</sup> study, the prevalence of *cagA* was 65.5% in Colombia and 100% in Japan, which showed that the prevalence of this gene in our study is similar to the Colombian isolates. In another study conducted in Bulgaria, the prevalence of *cagA* was 84.9% which is more than our results<sup>[26]</sup>. Interestingly, we had 6 isolates which had two different sizes of *cagA* simultaneously, showing the occurrence of mixed infection in these patients.

Variations of *vacA* are associated with different risks of gastrointestinal disorders. In general, *vacA* *s1* and *m1* genotypes produce a large amount of toxin, whereas *s2* and *m2* genotypes show little or no toxin production<sup>[27]</sup>. Recently, a third polymorphic determinant of vacuolating activity has been described as located between the s-region and m-region, an intermediate (i) region<sup>[28]</sup>. The frequency of the *vacA* *s1* and *vacA* *m1* genotypes in the Middle Eastern countries was found to be 71.5% and 32.8%, respectively<sup>[11]</sup>, which is in concordance with our study. We did not detect any *vacA* *s2m1* genotypes in our isolates which

has been reported to be rare<sup>[23]</sup>. The *vacA* *s1* and *m1* genotypes have been reported to be associated with *H. pylori*-related diseases; however *vacA* *s2* and *m2* strains are rarely associated with peptic ulcer and gastric cancer because of their low or non-vacuolating activities<sup>[23]</sup>. Genotyping of *vacA* will be useful in screening individuals for risk factors associated with gastric cancer and peptic ulcer development. Asrat *et al.*<sup>[29]</sup> showed that *vacAs1m1* genotype was the most common genotype in Ethiopian adult dyspeptic patients, and also *vacA*- and *cagA*-positive *H. pylori* strains were detected to a higher degree in patients with chronic active gastritis. Interestingly, similar to our results, correlation of the *vacA* *s1* genotype with the presence of the *cagA* gene was reported by Atherton<sup>[30]</sup>. The *vacAs1m2* genotype is more common in our Iranian patients, as previously described in Iran<sup>[31]</sup>. As reviewed by Suzuki *et al.*<sup>[32]</sup>, the predominant *vacA* genotypes in Asia, Europe and Africa are *vacAs1m1* and their subtypes, which is in contrast to our genotypes in Iranian isolates.

In spite of the low frequency of *vacAs1m1* genotypes in our study, isolates which carried the *vacAs1m1* gene showed significant associations with severe active chronic

gastritis. In a review by Hosseini *et al*<sup>[33]</sup>, they concluded that in contrast to *vacA*, there is no correlation between *cagA* genotype and disease status in the majority of studies conducted in Iran; but results of our study, however, proposed both of these genetic markers as useful indicators for predicting clinical outcomes in the studied population.

The meta-analysis by Shiota *et al*<sup>[8]</sup> confirmed the importance of the presence of *iceA* gene for peptic ulcer, although the significance was controversial. Such different results between the *iceA* allelic types and clinical disorders could be explained by the difference in geographic regions. In our study we found a significant relationship between *iceA*<sub>2</sub> genotype and clinical outcomes (severe active chronic gastritis), which was also observed by Caner *et al*<sup>[34]</sup> in Turkey. As Shiota *et al*<sup>[8]</sup> summarized in their meta-analysis, most of the studies showed no association between *iceA*<sub>1</sub> and *cagA* status, which is in concordance with our study. Interestingly, the prevalence of mixed genotype *iceA*<sub>1</sub> + *iceA*<sub>2</sub> (40.8%) in our study was higher than other studies which had detected this mixed genotype<sup>[35-37]</sup>. So this high prevalence with mixed genotypes makes it difficult to analyze potential relationships between the presence of each *iceA* allelic variant and clinical outcomes. *babA* genotype was frequently found in *H. pylori* strains in our study (95.8%); this was associated with severe active chronic gastritis. Although this genotype showed significant correlation with the existence of *cagA*, no significant correlation was observed with other virulence factors such as *vacA* *s*<sub>1</sub>/*s*<sub>2</sub>, *vacA* *m*<sub>1</sub>/*m*<sub>2</sub> and *iceA*<sub>1</sub>/*iceA*<sub>2</sub>. Chomvarin *et al*<sup>[38]</sup> detected the *babA* gene in 92% (103/112) of Thai patients, which is almost similar to our results; while in another study conducted in Cuba the prevalence of *babA* gene was lower (82.3%)<sup>[39]</sup>. It is important to mention that this PCR based method for *babA* genotyping must be confirmed by immunoblotting. Actually isolates were scored as *babA*-gene positive if the PCR and/or Southern blot analysis yielded a positive result<sup>[9]</sup>.

Regarding the combination of genotypes, we observed twenty different genotypes which showed vast diversities in the *H. pylori* isolates in our study. Interestingly there was not any significant association between these combined genotypes and clinical outcomes, except for *cagA*<sup>+</sup>/*vacA*<sub>1</sub>*m*<sub>1</sub>/*iceA*<sub>2</sub>/*babA*<sup>+</sup> genotype which showed significant association with severe active chronic gastritis.

Genotypes of *H. pylori*, especially *cagA* and *vacA*, are reported to be crucial factors determining the cure rates. So to select an *H. pylori* eradication regimen, we need to consider *H. pylori* genotypes<sup>[10]</sup>. *H. pylori* genotype distributions and their correlations with disease outcomes have shown geographical differences. In this regard, Yamaoka *et al*<sup>[7]</sup> reviewed that within East Asia, where the incidence of gastric cancer is high, that *vacA* *m*<sub>1</sub> genotype is dominant; whereas in southern parts where the gastric cancer incidence is low, the *m*<sub>2</sub> genotype, which we observed in our study, is predominant. Dabiri *et al*<sup>[31]</sup> showed that there was statistically no association between the *vacA*,

*cagA* and *cagE* status and clinical outcomes in Iranian patients, and recommended that other different markers may be more useful for this analysis. In comparison, in the current study, genotyping on the basis of *cagA*, *babA*, *vacA* and *iceA* was considered as a useful tool for predicting the clinical outcomes. Therefore, analyzing the multiple virulence factors of *H. pylori* (*cagA*, *vacA*, *iceA* and *babA*) might enable us to predict the patient's clinical outcome among Iranian patients. This prediction could be more accurate when accompanied by the impacts of environmental factors and host genetic polymorphisms such as interleukin-1 receptor antagonist gene polymorphism<sup>[37]</sup>. Nowadays, concurrent genotyping of *H. pylori* virulence markers and host factors is becoming increasingly crucial in the prediction of the diseases outcomes<sup>[40]</sup>.

In conclusion, our results show that most of the *H. pylori* isolates were highly virulent on the basis of the main clinically allelic variants in three or four virulence factors they carried. The Iranian isolates predominantly possessed different genotypes which showed vast diversities. Significant association of the noted genotypes with severe active chronic gastritis suggests that this genotyping panel is a suitable tool for detection of virulent *H. pylori* isolates that could provide valuable guidance for prediction of the clinical outcomes.

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## COMMENTS

### Background

Infection with *Helicobacter pylori* (*H. pylori*) causes diverse clinical outcomes such as persistent gastritis, peptic ulcers, mucosa associated lymphoid tissue lymphoma and gastric cancer. One of the reasons for these different clinical outcomes is genetic diversity of *H. pylori*; therefore determination of the pattern of *H. pylori* genotypes and its correlation with disease outcome, which shows geographic differences, is crucial.

### Research frontiers

The *H. pylori* genotyping may have multiple roles including prediction of clinical outcomes, impact on the *H. pylori* infection therapy, tracking human migration, and recently, the prediction of progression of gastric preneoplastic lesions. Therefore genotyping of *H. pylori* can be a valuable and multifunctional tool in the clinical field.

### Innovations and breakthroughs

In the majority of previous studies, the researchers were not able to detect any significant relationship between their genotyping panels and clinical outcomes for *H. pylori* infections. Most of these studies had used few genetic markers. In order to overcome this disadvantage, the authors have chosen greater numbers of *H. pylori* genetic markers for studying this association.

### Applications

The genotyping panel which contains eight important genetic markers can serve as a useful tool for typing of *H. pylori* isolates and, to some extent, predict clinical outcomes.

### Peer review

This is an epidemiological paper with statistical analysis, dealing with the important question of association between certain *H. pylori* genotypes and specific

pathologies, and with the problem of predictive value of *H. pylori* infection genotyping. In the submitted manuscript this issue is dissected in fine detail and uses quite extensive clinical material, thus providing novel and more reliable data.

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## Polymorphism in the interleukin-17A promoter contributes to gastric cancer

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### Abstract

**AIM:** To evaluate the contribution of the *G-197A* polymorphism in the interleukin-17 (IL-17) promoter region to gastric cancer risk in an Iranian population.

**METHODS:** We performed a case control study using samples from 161 individuals with gastric cancer and 171 healthy controls. For each individual, the *G-197A* genotype was determined by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified fragments. Statistical analyses were performed to determine whether any demographic or behavioral factors, infection with *Helicobacter pylori* (*H. pylori*), or a particular *G-197A* genotype was associated with gastric cancer risk.

**RESULTS:** We found that the *G-197A* genotype was

significantly associated with increased gastric cancer risk ( $P = 0.001$ ). Patients who were homozygous (AA) at position -197 were 2.9 times more likely to develop disease (95%CI: 1.56-5.4;  $P = 0.001$ ). Furthermore, logistic regression analysis revealed that the presence of a single A allele increased the risk of gastric cancer up to 1.7-fold (95%CI: 1.26-2.369;  $P = 0.001$ ). This association was observed for early stage gastric adenocarcinomas only, and was not linked to *H. pylori* infection.

**CONCLUSION:** These results suggest that carrying one or more *G-197A* polymorphisms at position -197 in the IL-17 promoter region significantly increases gastric cancer risk in this patient population.

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**Key words:** Gastric cancer; Interleukin-17A; Cancer; *Helicobacter pylori*

**Core tip:** There is currently a need for genetic markers to identify individuals at risk for developing gastric cancer. In this study, we describe one such marker, a *G-197A* polymorphism in the interleukin-17A (IL-17A) promoter. Within our study population, individuals who carry the *G-197A* polymorphism in the IL-17A promoter region may be at a significantly greater risk of developing gastric cancer. Importantly, the presence of this polymorphism alone was sufficient to increase risk of gastric cancer development.

Rafiei A, Hosseini V, Janbabai G, Ghorbani A, Ajami A, Farzmandfar T, Azizi MD, Gilbreath JJ, Merrell DS. Polymorphism in the interleukin-17A promoter contributes to gastric cancer. *World J Gastroenterol* 2013; 19(34): 5693-5699 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5693.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5693>

## INTRODUCTION

Gastric cancer is one of the most common causes of cancer-related deaths worldwide. Despite an overall decrease in gastric cancer incidence in recent years, this disease is still responsible for over 700000 deaths per year<sup>[1,2]</sup>, and represents a significant medical burden in many countries. In Northern Iran, gastric cancer has a major impact on public health due to the high morbidity and mortality associated with this disease. Indeed, several Iranian provinces, including Manzaradan, Semnan, Golestan, and the greater Tehran area, report age-standardized incidence rates for gastric cancer ranging from 25.4-49.1<sup>[3]</sup>. While these high incidence rates may be partially explained by the fact that a significant proportion of this population is also colonized by the carcinogenic bacterium *Helicobacter pylori* (*H. pylori*)<sup>[3,4]</sup>, the fact that this region maintains a high rate of gastric cancer despite an intensive *H. pylori* eradication program suggests that there are other host genetic and environmental factors involved in gastric cancer development.

Over the last several years, many studies have identified a variety of environmental, behavioral, and host genetic factors that play a role in gastric carcinogenesis across many patient populations. Among these behavioral factors are smoking and a high salt diet<sup>[5-8]</sup>, which have been shown to be particularly important for disease development in Northern Iran<sup>[6]</sup>. However, there is currently a lack of information regarding which host genetic factors may play a role in carcinogenesis in the Iranian patient population. Previous reports have identified a group of host immune factors that, when aberrantly expressed, can influence the development of gastric disease. Among these factors are the interleukin-1 (IL-1), IL-8, IL-10, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) genes, where specific polymorphisms have been associated with gastric cancer risk<sup>[9,10]</sup>. Additionally, in some instances, this effect can be compounded when the polymorphism exists in an *H. pylori* colonized individual. It is hypothesized that these polymorphisms result in a pro-inflammatory gastric environment, which may prime the tissue for cancer development.

Another, more recently described, pro-inflammatory cytokine is IL-17A (IL-17). This cytokine is one of a larger group of IL-17 family ligands and is primarily produced from a subset of CD4<sup>+</sup> effector cells known as Th17 cells<sup>[11-13]</sup>. IL-17 is involved in both innate and adaptive immunity and can act on a variety of cell types<sup>[11,12]</sup>. Recently, reports have indicated that certain *IL-17* polymorphisms are associated with autoimmune disease such as rheumatoid arthritis, graft *vs* host disease<sup>[14]</sup>, and inflammatory diseases such as ulcerative colitis<sup>[15]</sup>, suggesting that aberrant expression of this cytokine may polarize the body toward a variety of disease states. In addition, a recent study indicated that *H. pylori*-mediated induction of IL-17 may impact disease progression<sup>[16]</sup>; collectively, these studies highlight the importance of levels of IL-17 in a variety of diseases.

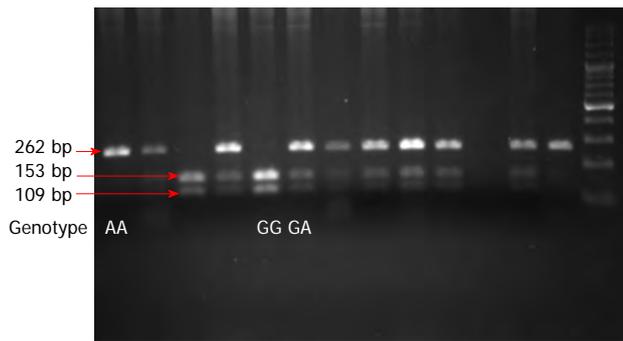
One particular *IL-17* polymorphism (G-197A or rs22759133) has also been associated with certain types of gastric cancer in both Japanese and Chinese populations<sup>[17,18]</sup>. The guanine to adenine substitution at position -197 within the *IL-17* promoter region is located in close proximity to 2 nuclear factors activated T cell binding motifs<sup>[19]</sup>. Because this region was shown to be required for *IL-17* expression<sup>[19]</sup>, it is believed that cells that harbor this mutation produce higher levels of *IL-17*, which in turn upregulates *IL-17*-mediated immune responses. This hypothesis is supported by the fact that various types of tumors express increased levels of *IL-17*<sup>[12]</sup>, and patients with gastric cancer have a greater number of circulating *IL-17*-producing Th17 cells than healthy controls<sup>[20]</sup>. Taken together, these findings highlight the potential role of *IL-17* in gastric cancer development.

Herein, we describe an epidemiologic study in which we investigate the role of the *IL-17* G-197A promoter polymorphism in gastric cancer risk among individuals from Northern Iran, which is traditionally a poorly studied population. We found that within this patient population the *G-197A* polymorphism was significantly more frequent in gastric cancer patients compared with controls. This association was independent of *H. pylori* colonization status. These data indicate that the *IL-17* G-197A polymorphism may be a good indicator for susceptibility to gastric cancer development in this patient population.

## MATERIALS AND METHODS

### Study participants

All aspects of the current study were approved by the Medical Research Ethics Committee at the Mandazaran University of Medical Sciences and conformed to the ethical guidelines set forth in the Declaration of Helsinki. Prior to enrollment, all patients were given an explanation of the nature of the study, and written informed consent was obtained from all individuals. Enrollees from the Mandazaran province of Iran were accepted after seeking treatment at Imam Teaching Hospital or Touba Polyclinic between April 2008 and November 2011. The diagnosis of gastric cancer cases were made based on gastric endoscopy, and cases were defined using the International Classification of Diseases for Oncology IX, Protocol 151 and Lauren criteria<sup>[21]</sup>. In order to simplify TNM staging<sup>[22]</sup>, Stages I A and I B were grouped into "Stage I", and Stages III A and III B were similarly combined into "Stage III". We enrolled a total of 161 patients with gastric cancer (89 male, 72 female), with a mean age of  $62.6 \pm 12.4$  years. One hundred seventy-one healthy controls (84 male and 87 female) were also enrolled, with a mean age of  $60.8 \pm 12.8$  years. Subjects within the control group were matched to the case group with respect to age, sex, ethnic background, and geographic origin. Demographics and behavioral and epidemiological risk factors were self-reported by study participants using a written questionnaire. Cigarette smokers were defined



**Figure 1 Interleukin-17 genotyping.** A representative image of the results of an interleukin-17 (IL-17) genotyping assay is shown. The IL-17 promoters were amplified by polymerase chain reaction and the resulting products were digested with Xag I. Products were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The *IL-17* GG genotype was evident as 153 and 109 bp fragments, GA as 262, 153 and 109 bp fragments, and AA as a single 262 bp fragment.

as those participants who reported smoking at least one cigarette per day for 12 mo. Consumption of salted fish, pickles, and fast food was defined as eating these items at least once a week for 6 mo.

#### *H. pylori* detection

All patients were tested for *H. pylori* infection using *H. pylori* specific IgG by ELISA (Diagnostic Automation, CA, United States) and by the urea breath test. Individuals that tested positive by either of these methods were considered as positive for *H. pylori*.

#### *IL-17* genotyping

Venous blood collected from all study participants was used to isolate genomic DNA restriction fragment length polymorphism analysis of polymerase chain reaction-amplified fragments (PCR-RFLP) as previously described<sup>[18]</sup>. Briefly, each PCR amplification was performed using 1  $\mu\text{mol/L}$  each of the forward (5'-ACAAGTA-AGAATGAAAAGAGGACATGGT-3') and reverse (5'-CCCCAATGAGGTCATAGAAGAATC-3') primers, 200  $\mu\text{mol/L}$  of each dNTP, 2 mmol/L MgCl<sub>2</sub>, 0.4 U of Hot Start Taq polymerase (Takara), 1X Takara Hot Start Taq PCR buffer, and 100 ng of genomic DNA in a final volume of 25  $\mu\text{L}$ . Each reaction was initially denatured at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 40 s, primer annealing at 60 °C for 35 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 4 min. Amplified PCR products were subjected to enzymatic digestion with XagI (Fermentas) for 12 h at 60 °C and visualized after separation by 3% sodium dodecyl sulfate polyacrylamide gel electrophoresis and staining with ethidium bromide. This procedure allowed us to clearly differentiate between the homozygous GG, heterozygous GA, and homozygous AA genotypes: the resulting restriction digest products from individuals with a homozygous (GG) genotype were 153 bp and 109 bp; digestion products from individuals who were heterozygous (GA) were 262 bp, 153 bp, and 109

**Table 1 Demographic data of the gastric cancer patients and healthy controls**

	Gastric cancer ( <i>n</i> = 161)	Control ( <i>n</i> = 171)	<i>P</i> -value <sup>1</sup>
Age (yr)	62.56 ± 12.44	60.81 ± 12.76	0.21
Sex (M/F)	89/72	84/87	0.16
Marital status			
Single	8 (5)	3 (1.8)	
Married	149 (92.5)	166 (97.1)	0.002
Divorced	4 (2.5)	2 (1.2)	
Occupation			
Unemployed	4 (2.5)	1 (0.6)	0.003
Employed	24 (14.9)	35 (20.5)	
Housewife	48 (29.8)	75 (43.9)	
Other	85 (52.8)	60 (35.1)	
Education 12 yr	53 (32.9)	85 (49.7)	0.003

<sup>1</sup>Significance of categorical variables was assessed using the  $\chi^2$  test; Differences in age were evaluated using the Student *t*-test. Percentages are shown in parentheses. "Other" is defined as an occupation that does not fall into one of the defined groups.

bp; a single 262 bp product was produced from individuals with a homozygous (AA) genotype (Figure 1).

#### Statistical analysis

After determining that all quantitative data were normally distributed (*via* Kolmogorov-Smirnov test), differences between patient populations were evaluated using the Student *t*-test. Qualitative differences between groups were assessed by the  $\chi^2$  test as indicated. The association between *IL-17* genotype and gastric cancer risk was determined using logistic regression analysis and an odds ratio (OR) with 95%CI. *P*-values  $\leq 0.05$  were considered significant for all tests.

## RESULTS

#### Patient demographics and epidemiology

The demographic data of gastric cancer patients and healthy controls are summarized in Table 1. Ages of study participants across the control group (*n* = 171) ranged from 24 to 87 years, and in the gastric cancer group (*n* = 161) from 28 to 86 years. The age difference between these 2 groups of participants was not statistically significant (*P* = 0.21). Similarly, the distribution of males and females in the study was also not significantly different between the gastric cancer group and the healthy controls (*P* = 0.16,  $\chi^2$  test). We did note a statistically significant difference in the distribution of married individuals in the cancer group and the healthy controls, where individuals in the gastric cancer group were more likely to be single or divorced (*P* = 0.002,  $\chi^2$  test). Similarly, we also noted that individuals within the gastric cancer group were more likely to be unemployed than those in the control group (*P* = 0.003,  $\chi^2$  test). Finally, we also detected a difference in the level of education between patients in the 2 groups; a significantly higher number of the healthy controls had > 12 years of education compared with the gastric cancer patients (*P* = 0.003,  $\chi^2$  test).

**Table 2** Frequency of the distribution of the *G-197A* (rs2275913) polymorphism of the interleukin-17A gene in gastric cancer patients and healthy controls *n* (%)

Genotype	Cancer ( <i>n</i> = 161)	Controls ( <i>n</i> = 171)	OR	CI	<i>P</i> -value <sup>1</sup>
GG	56 (34.8)	78 (45.6)	1.00 <sup>2</sup>		
GA	61 (37.9)	72 (42.1)	1.2	0.73-1.91	0.53
AA	44 (27.3)	21 (12.3)	2.92	1.56-5.4	0.001
G allele	173 (53.7)	228 (66.7)	1.00 <sup>2</sup>		
A allele	149 (46.3)	114 (33.3)	1.72	1.26-2.36	0.001
A allele carriage (AA + GA vs GG)	105 (64.2)	93 (54.4)	1.57	1.01-2.45	0.04

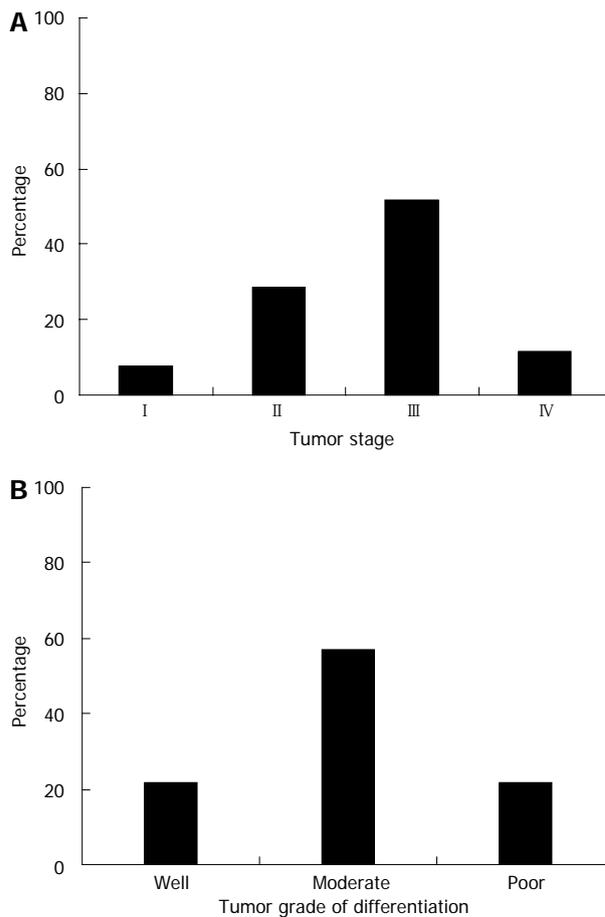
Genotype frequencies are indicated in absolute values, with the percentage in parentheses. G allele and A allele indicates the total number of each individual allele within each group. <sup>1</sup>Two-sided  $\chi^2$ -test; <sup>2</sup>The first allele or genotype is considered as the reference for this analysis. OR: Odds ratio.

**Frequency and distribution of IL-17 genotypes and gastric cancer risk**

We next evaluated the distribution of the IL-17-197 alleles between the 2 study groups essentially as previously described<sup>[17,18]</sup>. The genotype frequencies of this polymorphism in controls were within the Hardy-Weinberg equilibrium (*P* = 0.49). As shown in Table 2, the predominant genotype found in gastric cancer patients was the heterozygous GA allele (38%), followed by the homozygous alleles GG (35%) and AA (27%). In contrast, within the healthy control group, the most common genotype was the wildtype GG allele (45.6%) followed by the heterozygous GA allele (42%) and the homozygous AA allele (12%). While the difference in the distribution of the GG and GA genotypes between the gastric cancer and control groups was not statistically significant, the finding that a larger number of cancer patients carried the AA allele was significant (*P* = 0.001,  $\chi^2$  test). There was also a significant difference in the frequency of the A allele between the 2 groups; this allele was present in 46% of gastric cancer patients compared with only 33% of healthy controls (*P* = 0.001,  $\chi^2$  test). We next performed a multivariate regression analysis to determine the predictive value of the *G-197A* polymorphism for gastric cancer development. After correcting for covariates such as age, sex, and *H. pylori* infection, this analysis indicated that the presence of the A allele increased gastric cancer risk by 1.7-fold (95%CI: 1.26-2.36; *P* = 0.001). The presence of the AA mutant genotype increased the odds of developing gastric cancer up to 2.9-fold (95%CI: 1.56-5.4; *P* = 0.001), indicating that the presence of the AA genotype at this locus is significantly associated with increased gastric cancer risk. Additionally, harboring the allele (AA + GA) enhanced the risk of gastric cancer up to 1.6-fold (95%CI: 1.01-2.45; *P* = 0.04).

**Effect of G-197A polymorphism and cancer progression**

In order to determine whether the presence of the -197A allele was associated with disease progression within the gastric cancer group, we stratified a subset of the patients from this group based on TNM staging, extent of tumor



**Figure 2** Tumor, node, metastasis staging, and cellular differentiation in the gastric cancer population. A: Breakdown of tumor staging among a subset of the gastric cancer population. Staging was categorized as described in the Materials and Methods section. For simplicity, individuals that were categorized as Stage I A or I B were grouped into Stage I. Similarly, patients with Stage III A or III B tumors were grouped into Stage III; B: The degree of cellular differentiation seen in patient tumors was graded as well, moderate, or poor as described in the Materials and Methods.

cell differentiation, and the presence or absence of the mutant A allele (AA + GA vs GG); TNM information for 77 of the 161 patients enrolled in the gastric cancer group was available. A breakdown on tumor staging and degree of cellular differentiation are shown in Figure 2A and B, respectively. We placed individuals with lower grade malignancies (Stage I or II) in one group (*n* = 28), and those with Stage III or IV malignancies into a second group (*n* = 49) (Table 3). Within the group with Stage I or II malignancies, 22 patients had at least one A allele (GA or AA genotype), while only 6 patients had the GG genotype. This difference in Stage I / II patients was statistically significant (*P* = 0.001,  $\chi^2$  test). Furthermore, the presence of the 197A allele increased the risk of gastric cancer development at the early stages of tumorigenesis by 6.3-fold (95%CI: 2.2-18.56; *P* = 0.001). In contrast, this association was not observed in patients with Stage III/Stage IV malignancies or when we grouped the cancer patients by age, sex, *H. pylori* status, or tumor cell differentiation (Table 3). These data suggest

**Table 3** Effect of *G-197A* polymorphism on gastric cancer development *n* (%)

	GA + AA	GG	OR	95%CI	P-value <sup>2</sup>
Age					
< 50 yr	18 (17.1)	4 (7.1)	2.7	0.8-8.4	0.09
≥ 50 yr	87 (82.9)	52 (92.9)			
Gender					
Male	51 (48.6)	21 (37.5)	1.6	0.8-3.05	0.19
Female	54 (51.4)	35 (62.5)			
<i>H. pylori</i> +	65 (61.9)	33 (58.9)	0.88	0.45-1.71	0.74
<i>H. pylori</i> -	40 (38.1)	23 (41.1)			
TNM stage <sup>1</sup>					
I - II	22 (55)	6 (16.2)	6.3	2.2-18.5	0.001
III-IV	18 (45)	31 (83.8)			
Tumor differentiation					
Well	25 (23.8)	9 (16.1)	1.00 <sup>3</sup>		
Moderate	57 (54.3)	35 (62.5)	0.56	0.24-1.34	0.19
Poor	23 (21.9)	12 (21.4)	0.66	0.23-1.86	0.43

<sup>1</sup>Data presented for 77 patients; <sup>2</sup>All comparisons between categorical variables were made using a two-sided  $\chi^2$  test; <sup>3</sup>Used as reference for tumor differentiation analyses. Values in parentheses indicate the percentage. *H. pylori*: *Helicobacter pylori*; TNM: Tumor, node, metastasis.

that while the presence of a mutant A allele at this locus increased the risk of developing a low grade (Stage I or II) malignancy, it was not a risk factor for progression to later stage cancer (Stage III or IV).

## DISCUSSION

Gastric cancer remains a significant source of morbidity and mortality worldwide. As such, being able to identify which patients or patient populations are most at risk for developing this severe disease is of the utmost importance. This fact is particularly true for geographical regions such as Iran that have exceptionally high disease rates<sup>[23]</sup>. Indeed, despite the alarmingly high rates of gastric cancer in this region, few studies have focused on the identification of host factors or mutations in these factors that may predispose members of this population to gastric cancer development. Once identified, these factors or mutations could then be exploited to aid in the diagnosis of high-risk patients.

Numerous studies have attempted to unravel the complex nature of gastric cancer development. From these studies it has become clear that carcinogenesis is a multi-factorial process that involves a combination of environmental/behavioral, and genetic factors. For many populations/geographic areas, including the focus of the current study, major environmental/behavioral risk factors for gastric cancer development have been identified<sup>[2,5-8]</sup>. Additionally, there have been many studies that have identified potential genetic markers or polymorphisms that are associated with gastric cancer risk. Several of these factors play a role in maintaining proper immune homeostasis, including the pro-inflammatory cytokines IL-1 $\beta$ , inducible nitric oxide synthase, TNF- $\alpha$ , IL-8, IL-10<sup>[9,10,24]</sup>, and more recently IL-17<sup>[17,18]</sup>. However, since many of these factors have only been studied in

limited patient populations, it remains unclear whether or not the prognostic value of these markers applies equally to all groups. In fact, as more studies are performed across a variety of patient populations, it has become evident that the degree to which these factors impact on disease development is often dependent upon the group being studied<sup>[19,25-28]</sup>. As a result, there is a need to examine the role of these factors in additional populations.

Here, we described a case-control study that examined the association of the *G-197A* IL-17 promoter mutation with gastric cancer development in an Iranian population. This particular polymorphism has been previously associated with an increased risk of gastric mucosal atrophy and gastric cancer in a Japanese population<sup>[17]</sup>, as well as gastric cancer risk in a Chinese population<sup>[18]</sup>. In accordance with those studies, we found that the *G-197A* polymorphism is significantly associated with an increase in gastric cancer risk (Table 2). Specifically, harboring 2 copies of the mutant allele (AA) at this locus increased a patient's likelihood of developing cancer by a factor of 2.8 (Table 2). Furthermore, harboring only a single copy of this polymorphism (a heterozygous GA genotype) increased gastric cancer risk by 1.5 fold; this finding suggests that the effect of this polymorphism follows a dose-response. These data are consistent with the previous finding that the effect of the *G-197A* polymorphism on inflammation follows a similar dose-response pattern<sup>[17]</sup>.

In healthy individuals, IL-17 is involved in both innate and adaptive arms of the immune system. Specifically, IL-17 is involved in induction of other pro-inflammatory cytokines as well as the recruitment and activation of inflammatory cells such as neutrophils and macrophages<sup>[11,29]</sup>. As the receptor for this cytokine is widely distributed on intestinal epithelial cells<sup>[12]</sup> and other tissue types<sup>[29]</sup>, changes in the levels of IL-17 expression may have far reaching effects. This fact is illustrated by several studies, which have implicated increased IL-17 production with a variety of pathologic processes. Indeed, increased IL-17 transcript levels have been detected in patients with coronary artery disease<sup>[30]</sup>, and inflammatory bowel disease<sup>[31]</sup>, and specific *IL-17* polymorphisms have been associated with ulcerative colitis<sup>[15]</sup>, rheumatoid arthritis<sup>[32]</sup>, and graft *vs* host disease<sup>[14]</sup>. While these conditions may present quite differently from gastric cancer, the underlying commonality among these diseases is their inflammatory origin.

While the precise mechanistic role of the *G-197A* polymorphisms in gastric cancer development remains unclear, a plausible hypothesis is that increased/constitutive expression of IL-17 may skew the gastric environment to become pro-inflammatory. As chronic inflammation is a known precursor for gastric cancer development<sup>[33]</sup>, this IL-17-mediated inflammatory environment may result in an increase in carcinogenic cellular damage, which predisposes an individual to develop disease. Once these initial steps have begun, the cancer may progress in an IL-17 dependent or independent manner. In the current study, we found that the -197A allele was

only significantly associated with the development of lower grade malignancies (Stage I or II) (Table 3). Similarly, a previous study linked the -197A polymorphism to an increased risk of poorly differentiated TNM Stage I / II cancer<sup>[18]</sup>. These data perhaps suggest that progression to more severe disease (Stages III or IV) occurs at least partially in an IL-17-independent manner. However, as 18 of the 49 TNM Stage III or IV cancer patients (Table 3) carried at least one mutant allele, we cannot completely rule out the possibility that this *IL-17* polymorphism impacts on disease progression to some extent.

Gastric cancer remains a global health problem. As diagnosis of this disease often occurs only after the progression to more severe stages, there is a serious need for diagnostic markers that could help preemptively screen patients in high-risk populations and identify those who are most at risk of developing disease. Because the *G-197A* polymorphism in the *IL-17* promoter region is consistently linked to gastric cancer development in multiple populations, it may be a good global candidate marker to identify gastric cancer risk.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Individuals who carry specific genetic polymorphisms can be prone to cancer development. As a result, these polymorphisms may be used to identify these at risk individuals. However, before a particular polymorphism can be reliably used as a marker for cancer risk, the link between the polymorphism and disease propensity must be verified in a multiple populations of people with diverse genetic backgrounds.

### Research frontiers

Interleukin-17 (IL-17) is an important pro-inflammatory cytokine that is involved in both the innate and adaptive arms of the human immune system. One of the research hotspots in the field of IL-17 research is determining how increased or decreased levels of this cytokine effect human physiology and disease development.

### Innovations and breakthroughs

Previous studies have identified the *G-197A IL-17* polymorphism as a potential genetic marker for gastric cancer risk. However, those studies were performed on a limited population that had a similar genetic background. The current study verified the *G-197A* polymorphism as a potential genetic marker for gastric cancer risk in a genetically distinct population. Their results reinforce the possibility of using this *IL-17* polymorphism as a marker for disease risk in many diverse populations.

### Applications

The study highlights the possibility that the *IL-17 G-197A* polymorphism could be used as a marker for gastric cancer risk across diverse populations.

### Terminology

A polymorphism is where multiple forms of a DNA sequence may be present at a single genetic site.

### Peer review

The manuscript is quite well written. The results justify the conclusions drawn.

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## Chronological changes in the liver after temporary partial portal venous occlusion

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non-obstructed lobe, there were no significant differences within each group. The duration of PV occlusion did not seem to be strong enough to introduce liver weight increase. Stimulation of liver regeneration was brought about in the non-occluded lobe by 12-h occlusion, and was sustained even at 48 h after reperfusion. The obstructed lobe atrophied with the passage of time in the obstructed state. However, the proliferating-cell nuclear antigen labeling index also increased at 48 h after reperfusion, and a repair mechanism was observed.

**CONCLUSION:** Temporary blood flow obstruction of the portal vein may become a significant trigger for liver regeneration, even with an obstruction of 12 h.

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**Key words:** Temporary; Portal vein; Occlusion; Regeneration; Liver

### Abstract

**AIM:** To investigate time-dependent changes caused by temporal portal vein obstruction and subsequent reperfusion in the lobe with or without an occluded portal vein.

**METHODS:** The portal vein (PV) of the anterior lobe of the liver of a male Wistar rat (8 wk-old) was obstructed (70%) for 12, 24, 36 and 48 h, respectively, and models were sacrificed at 48 h after reperfusion (each group:  $n = 10$ ). The histological changes and the status of liver regeneration were compared between a liver biopsy performed on each lobe after temporary obstruction of the portal vein in the same rat liver, and the liver extracted at the time of sacrifice (48 h after reperfusion).

**RESULTS:** With regard to the obstructed lobe, the liver weight/body weight ratio significantly decreased according to obstruction time. On the other hand, in the

**Core tip:** This paper describes the chronological effects of temporary portal venous branch ligation on liver regeneration in rats. These results imply that, in the future, it might be possible to control liver regeneration. In the clinical setting, we have just completely occluded the portal venous branch irreversibly.

Hamasaki K, Eguchi S, Soyama A, Hidaka M, Takatsuki M, Fujita F, Kanetaka K, Minami S, Kuroki T. Chronological changes in the liver after temporary partial portal venous occlusion. *World J Gastroenterol* 2013; 19(34): 5700-5705 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5700.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5700>

### INTRODUCTION

Permanent obstruction of the portal vein, as clinically

applied in portal branch ligation (PBL) or percutaneous transhepatic portal venous embolization, evokes liver regeneration<sup>[1-4]</sup>. This technique enables relatively major hepatic resection for malignancy in an occluded liver lobe<sup>[5-9]</sup>. In addition, PBL has been used to induce a regenerative stimulus for transplanted hepatocytes or pancreatic islet cells for cell therapy<sup>[10,11]</sup>.

Although short term temporary occlusion can induce some degree of liver regeneration, an investigation of liver regeneration caused by temporary portal vein obstruction, as well as time-dependent changes resulting from reperfusion, has not yet been performed<sup>[12]</sup>. Therefore, the current study aimed to examine time-dependent changes in a lobe with a portal vein occlusion of an unobstructed portal vein caused by temporary portal vein obstruction and reperfusion as the central focus.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (200-240 g, Japan SLC Inc., Shizuoka, Japan) were used for the experiments. All animals were maintained at 24 °C with a 12-h light-dark cycle and given free access to tap water and standard laboratory chow. The animals were treated in accordance with the guidelines stated in the University of Nagasaki Research Animal Resources during all experimental procedures.

### Experimental design

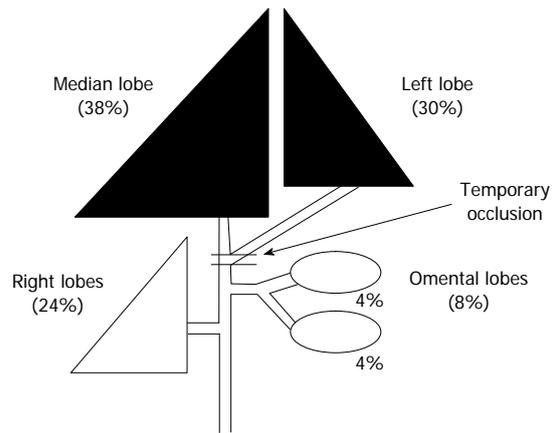
The portal vein of the anterior lobe (medial and left lobes) of the liver of a male Wistar rat (8 wk old) was occluded (70%) for 12, 24, 36 and 48 h, respectively (Figure 1). Rats to be sacrificed were prepared at 48 h after each reperfusion (models for each group:  $n = 10$ , Figure 2). The histological changes over time and the status of liver regeneration were compared between liver biopsies performed from each lobe after temporary obstruction of the portal vein in the same rat liver, and the liver extracted at the time of sacrifice (48 h after reperfusion).

### Liver to body weight ratio

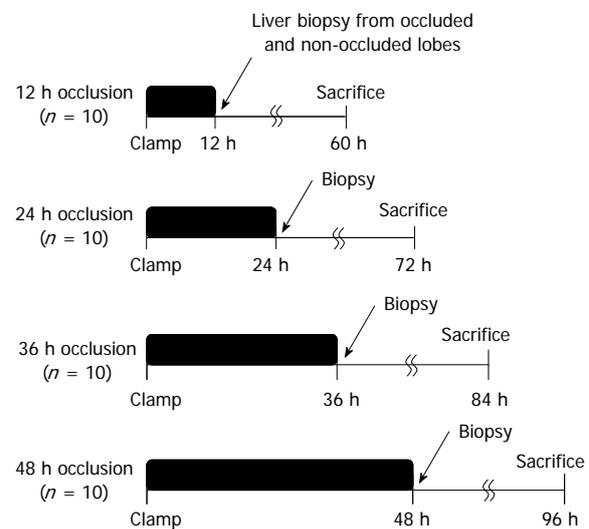
The body weights and liver weights were recorded following the sacrifice of the rats to compare the rate of liver regeneration. The liver weight was expressed as a percentage of the body weight (%) and used as an index.

### Histology and immunohistochemistry

Formalin-fixed paraffin embedded (4  $\mu\text{m}$ ) sections were used for hematoxylin-eosin (HE) staining. Proliferating-cell nuclear antigen (PCNA) immunostaining was performed to examine hepatocyte proliferation using a mouse monoclonal antibody against PCNA (clone-PC 10; Dako, Kyoto, Japan)<sup>[13]</sup>. Briefly, liver tissue specimens were fixed in 10% buffered formalin, embedded in paraffin and then cut into 5  $\mu\text{m}$  sections. The deparaffinized sections were heated in a microwave three times in phosphate-buffered saline (PBS) for 5 min each and were then washed three times with PBS for 5 min each. After blocking endogenous peroxidase activity, the specimens



**Figure 1** Schematic drawing of the rat model. The portal vein of the anterior lobe (black area) of the liver was occluded (68%) for various durations, while the arteries remained open.



**Figure 2** Experimental protocol. The portal vein of the anterior lobe (median and left liver lobes) of the liver of a male Wistar rat was occluded (70%) for 12, 24, 36 and 48 h. Rats to be sacrificed were prepared at 48 h after each reperfusion (models for each group:  $n = 10$ ).

were washed three times with PBS for 5 min each. The sections were incubated with an antibody against PCNA overnight at 4 °C. After washing several times with PBS, biotin-labeled secondary antibody was added for 1 h at room temperature. After washing several times with PBS, the tissue peroxidase activity was visualized using diaminobenzidine.

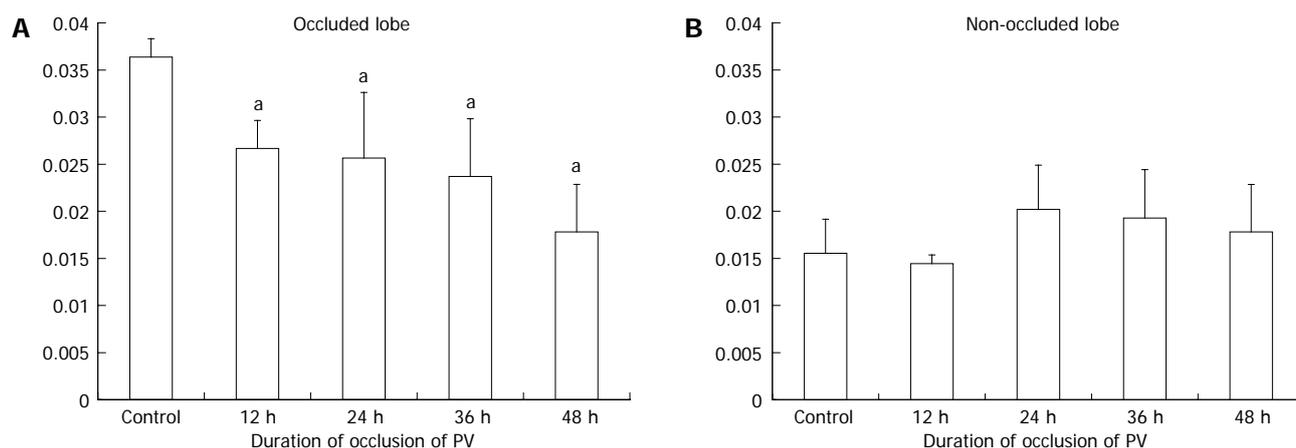
The PCNA labeling index (PCNA LI) was then determined as the number of PCNA-positive cells among 1000 counted cells.

### Statistical analysis

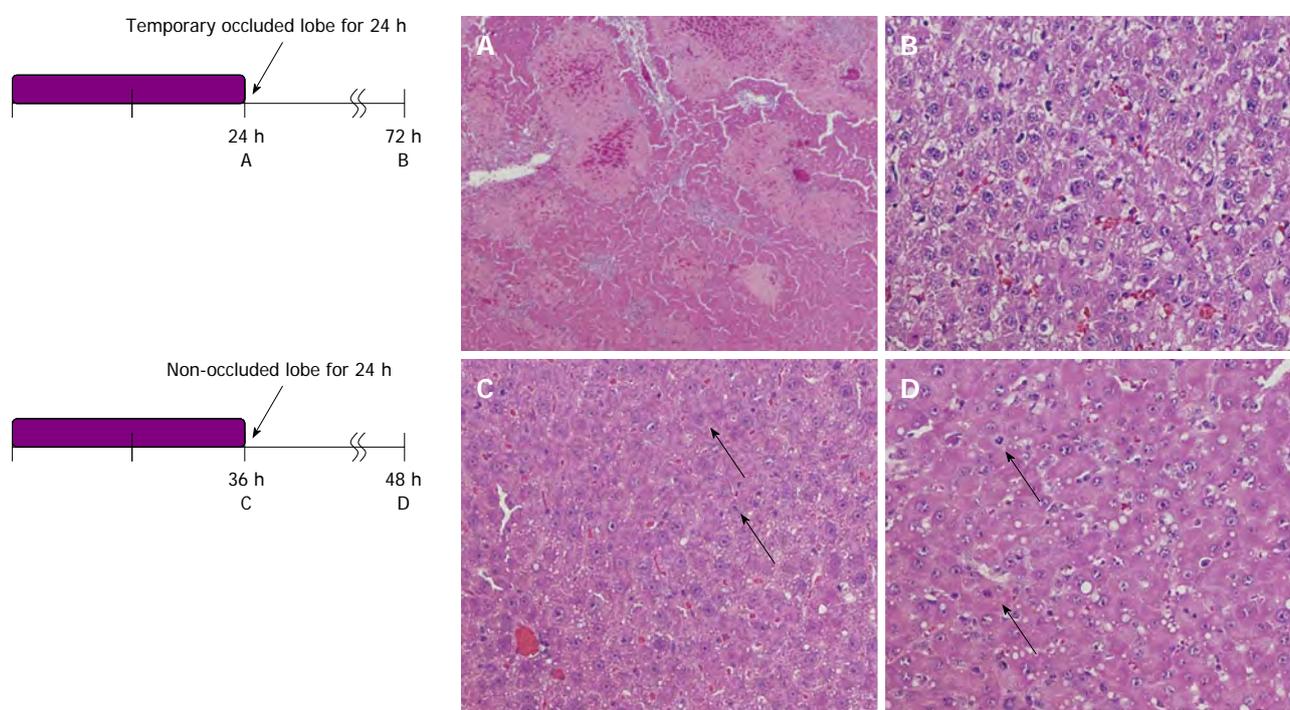
All of the data were expressed as the mean  $\pm$  SD. The Mann-Whitney *U*-test was used for data analysis. A level of  $P < 0.05$  was considered statistically significant.

## RESULTS

With regard to the obstructed lobe, the liver weight/body



**Figure 3 Liver weight/body weight.** A: The liver weight/body weight ratio was decreased by temporary occlusion of the occluded anterior lobes; B: There were no significant differences within each group in non-occluded lobe. <sup>a</sup> $P < 0.05$  vs control group.



**Figure 4 Changes in hepatic histology.** Histology in temporary occluded lobes. A: 24 h occlusion,  $\times 100$ ; B: 36 h occlusion,  $\times 400$ ; C: In non-occluded lobes 24 h occlusion,  $\times 100$ ; D: 48 h occlusion,  $\times 400$  is shown by HE staining. In the occluded liver lobe, before reperfusion, coagulative necrosis may be observed around the central vein in proportion to the occlusion time. However, the above-mentioned necrotic area decreased at 48 h after portal vein reperfusion. In the non-occluded liver lobe, hepatocytes became hypertrophic, and some mitoses could be observed (arrows).

weight ratio significantly decreased with increasing obstruction time (Figure 3). On the other hand, in the non-obstructed lobe, there were no significant differences within each group. The duration of PV occlusion did not seem to be strong enough to induce an increased in liver weight.

Liver histology was investigated under HE staining (Figure 4). In the occluded liver lobe, before reperfusion, coagulative necrosis was observed around the central vein in proportion to the occlusion time. However, after 48 h of reperfusion, the above-mentioned necrotic area

decreased. On the other hand, in the non-occluded liver lobe, hepatocytes became hypertrophic, and some mitoses were observed.

In the non-obstructed lobe, there were no significant differences in the PCNA LI within each group (Figure 5). LI seemed to peak at 36 h of biopsy (non-obstructed models at 12, 24, 36 and 48 h = 24%, 32%, 36% and 31%, Figure 5C). In the non-occluded lobe at 48 h after reperfusion (models for each group = 33%, 32%, 36% and 32%), the PCNA LI was still significantly increased at all points compared with the control (Figure 5D).

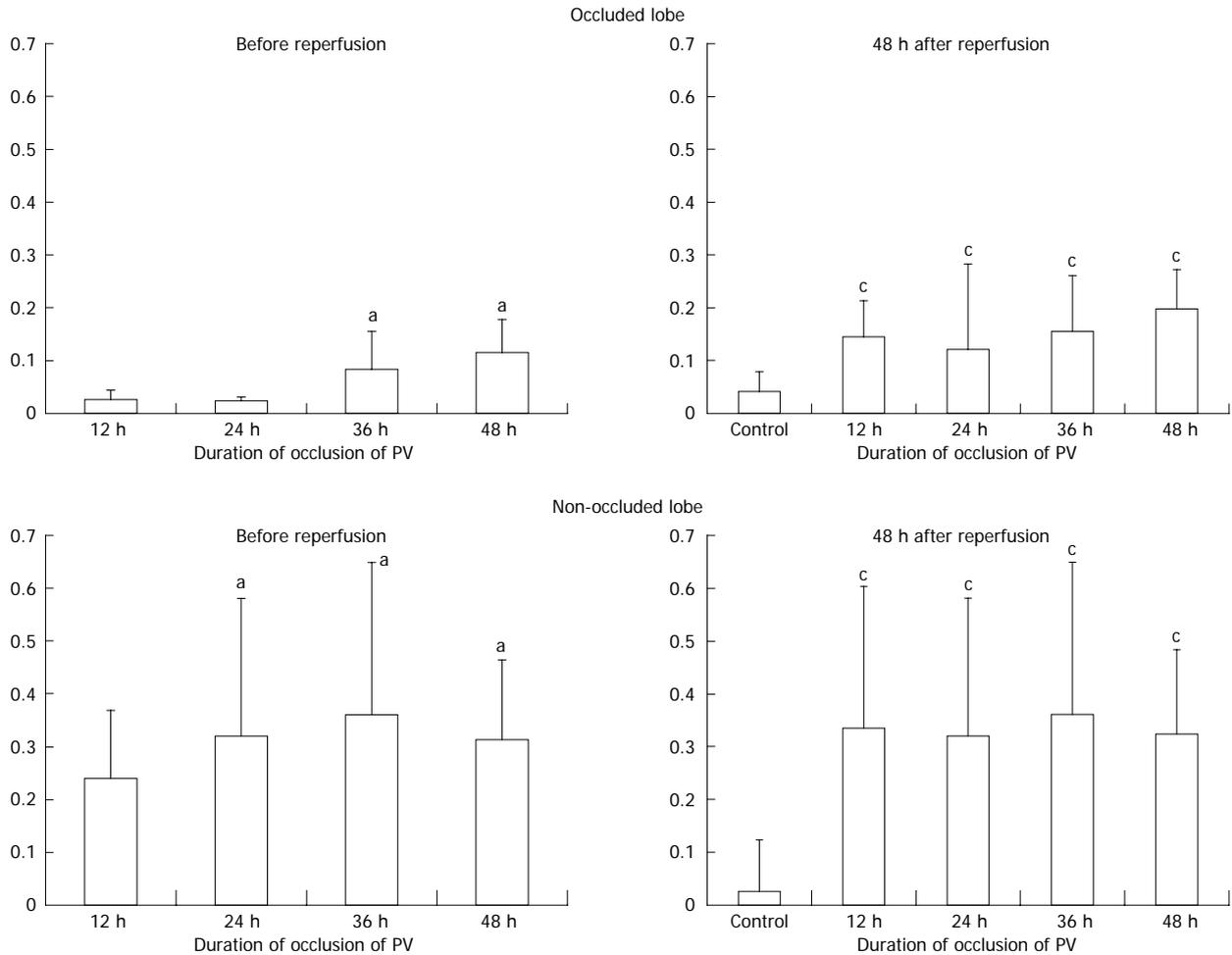


Figure 5 Proliferating-cell nuclear antigen labeling index. Proliferating-cell nuclear antigen (PCNA) labeling index in each lobe at the time before reperfusion and 48 h after reperfusion. <sup>a</sup>*P* < 0.05 vs 12 h group; <sup>c</sup>*P* < 0.05 vs 24 h group. PV: Portal vein.

On the other hand, hardly any positive cells were observed in a biopsy of the obstructed lobe (obstructed models at 12, 24, 36 and 48 h = 2%, 2%, 8% and 10%). However, at 48 h after reperfusion, an increase in LI was observed (models for each group = 14%, 12%, 15% and 19%).

## DISCUSSION

Portal venous branch ligation or embolization (PBL or PBE) can induce atrophy of the ligated lobe, while inducing hypertrophy of a non-ligated lobe, which enables extended hepatectomy for a malignant tumor in a ligated lobe<sup>[14-16]</sup>. In addition, PBL has been used as a regenerative stimulator to induce transplanted cell proliferation in animal models for hepatocyte-based cell therapy<sup>[17-23]</sup>. The length of time of occlusion needed to induce remnant liver regeneration, *i.e.*, temporary portal venous occlusion, remains unknown. In the present study, the stimulation of liver regeneration brought about in the non-occluded liver lobe was sustained, even after 48 h from reperfusion. Thus, temporary blood flow obstruction of the portal vein may be a significant trigger of liver regeneration, with an obstruction of at least 12 h. However, the

duration of PV occlusion in this study did not seem to be long enough to induce an increase in liver weight.

Interestingly, there was no significant difference in PCNA LI in the non-occluded lobe according to the duration of portal vein occlusion up to 48 h. Therefore, in the clinical setting, the same extent of liver hypertrophy may be induced with temporary balloon occlusion in as short as 12 h, to minimize an invasive procedure, although there might be difference among species.

As a cell therapy, many investigator have used liver for the engraftment of many cell types<sup>[10-12,14-16]</sup>. In fact, transplanted cells (hepatocytes, pancreatic islet cells or genetically engineered cells) could be induced to proliferate using temporary portal venous occlusion. Although there must be some differences between humans and rodents in terms of liver regenerative activity, our results provide a new insight into temporary stimulation of liver regeneration for subsequent treatment procedures<sup>[24-26]</sup>.

On the other hand, the PCNA labeling index of the obstructed lobe was also increased 48 h after portal venous reperfusion. This could be a repair mechanism for portal vein ischemia in the occluded lobe, although PCNA LI was lower compared to that in the non-occluded lobe that undergoes liver regeneration<sup>[14]</sup>. Although it

was not observed up to 48 h, this result of the temporary occlusion of the lobe provided an interesting phenomena; however, the lack of temporal portal venous flow could become atrophic if portal venous ischemia had lasted longer. The duration for the “point of no return” should be investigated in further research.

In conclusion, a temporary blood flow obstruction of the portal vein may be a significant trigger for liver regeneration, even with an obstruction of 12 h. The histological changes in the unobstructed lobe and obstructed lobe in cases of temporary blood flow obstruction of the portal vein and at 48 h after reperfusion were described.

## COMMENTS

### Background

Permanent obstruction of the portal vein, as clinically applied in portal branch ligation (PBL) or percutaneous transhepatic portal venous embolization, evokes liver regeneration. This technique enables relatively major hepatic resection for malignancy in an occluded liver lobe. In addition, PBL has been used to induce a regenerative stimulus for transplanted hepatocytes or pancreatic islet cells for cell therapy.

### Research frontiers

Portal venous branch ligation or embolization can induce atrophy of the ligated lobe, while inducing hypertrophy of a non-ligated lobe, which enables extended hepatectomy for a malignant tumor in a ligated lobe. In addition, PBL has been used as a regenerative stimulator to induce transplanted cell proliferation in animal models of hepatocyte based cell therapy. The length of time of occlusion required to induce remnant liver regeneration, *i.e.*, temporary portal venous occlusion, remains unknown.

### Innovations and breakthroughs

The histological changes and the status of liver regeneration were compared between a liver biopsy performed on each lobe after temporary obstruction of the portal vein in the same rat liver, and the liver extracted at the time of sacrifice (48 h after reperfusion).

### Peer review

This research is very important because it shows that a temporary obstruction of the portal vein as a trigger of liver regeneration. Recently, it has been used as a regenerative stimulator to induce transplanted cell proliferation. Thus, the manuscript approached an interesting subject from the surgical and scientific points of view; however, several aspects should be better evaluated.

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**S- Editor** Gou SX **L- Editor** Stewart GJ **E- Editor** Zhang DN



## Efficacy of treatment with rebamipide for endoscopic submucosal dissection-induced ulcers

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Author contributions: Takayama M and Matsui S contributed equally to this work; Takayama M, Matsui S, Kawasaki M, Asakuma Y, Sakurai T, Kashida H and Kudo M designed the research; Takayama M and Matsui S performed the research; Takayama M and Matsui S analyzed the data; Takayama M wrote the paper.

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### Abstract

**AIM:** To prospectively compare the healing rates of endoscopic submucosal dissection (ESD)-induced ulcers treated with either a proton-pump inhibitor (PPI) or rebamipide.

**METHODS:** We examined 90 patients with early gastric cancer who had undergone ESD. All patients were administered an intravenous infusion of the PPI lansoprazole (20 mg) every 12 h for 2 d, followed by oral administration of lansoprazole (30 mg/d, 5 d). After 7-d treatment, the patients were randomly assigned to 2 groups and received either lansoprazole (30 mg/d orally,  $n = 45$ ; PPI group) or rebamipide (300 mg orally, three times a day;  $n = 45$ ; rebamipide group). At 4 and 8 wk after ESD, the ulcer outcomes in the 2 groups were compared.

**RESULTS:** No significant differences were noted in patient age, underlying disease, tumor location, *Helicobacter pylori* infection rate, or ESD-induced ulcer

size between the 2 groups. At both 4 and 8 wk, the healing rates of ESD-induced ulcers were similar in the PPI-treated and the rebamipide-treated patients (4 wk: PPI, 27.2%; rebamipide, 33.3%;  $P = 0.5341$ ; 8 wk: PPI, 90.9%; rebamipide, 93.3%;  $P = 0.6710$ ). At 8 wk, the rates of granulation lesions following ulcer healing were significantly higher in the PPI-treated group (13.6%) than in the rebamipide-treated group (0.0%;  $P = 0.0103$ ). Ulcer-related symptoms were similar in the 2 treatment groups at 8 wk. The medication cost of 8-wk treatment with the PPI was 10945 yen *vs* 4889 yen for rebamipide. No ulcer bleeding or complications due to the drugs were observed in either treatment group.

**CONCLUSION:** The healing rate of ESD-induced ulcers was similar with rebamipide or PPI treatment; however, rebamipide treatment is more cost-effective and prevents granulation lesions following ulcer healing.

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**Key words:** Early gastric cancer; Rebamipide; Endoscopic submucosal dissection; Gastric ulcer; Proton-pump inhibitor

**Core tip:** In this prospective randomized, parallel-controlled study, we demonstrated that rebamipide monotherapy was as effective as proton-pump inhibitor (PPI) in the healing of endoscopic submucosal dissection-induced ulcers, regardless of the location of the resected cancer, the degree of atrophic gastritis, or the presence of *Helicobacter pylori* infection. In addition, rebamipide treatment is more cost-effective and results in a better quality of ulcer healing compared with the PPI lansoprazole.

Takayama M, Matsui S, Kawasaki M, Asakuma Y, Sakurai T, Kashida H, Kudo M. Efficacy of treatment with rebamipide for endoscopic submucosal dissection-induced ulcers. *World J Gastroenterol* 2013; 19(34): 5706-5712 Available from: URL:

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<http://dx.doi.org/10.3748/wjg.v19.i34.5706>

## INTRODUCTION

Endoscopic mucosal resection (EMR) is a well-established curative treatment for gastric neoplasms, such as early gastric cancer confined to the mucosa. However, EMR, performed using conventional techniques such as strip biopsy or cap EMR, does not always achieve *en bloc* resection. Thus, endoscopic submucosal dissection (ESD) has become the preferred treatment method. Compared with EMR, ESD facilitates the collection of larger specimens, regardless of lesion size or location, resulting in a higher rate of *en bloc* and histologically complete resection. Moreover, the rate of local recurrence of tumors after ESD may be lower than that after conventional EMR<sup>[1]</sup>. However, the iatrogenic ulcer that develops as a result of ESD is large, and requires a considerably longer healing time compared to that resulting from conventional EMR.

Proton-pump inhibitors (PPIs) are the most effective medications for the treatment of ESD-induced ulcers. However, studies have shown that PPI monotherapy does not heal the ESD-induced ulcers sufficiently within 4 wk<sup>[2-6]</sup>. Increased understanding of the mucosal defense system has prompted the development of mucoprotective agents for clinical use in Japan. The efficacy of combination treatment involving PPIs and the mucoprotective agent rebamipide in the early treatment of ESD-induced ulcers and in the prevention of relapse of such disorders has been clearly indicated<sup>[2-5]</sup>.

Rebamipide [2-(4-chlorobenzoylamino)-3-[2-(1H)-quinolinon-4-yl]-propionic acid], a novel mucosal-protective and ulcer-healing drug, is widely prescribed in East Asia. Previous studies have indicated that rebamipide is effective in the treatment of gastric ulcers as well as decreasing the recurrence rate, without affecting the *Helicobacter pylori* infection status of the patients<sup>[7-12]</sup>. In addition, previous randomized-controlled studies have also found that rebamipide can prevent the formation of peptic ulcers induced by the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) and can suppress the mucosal inflammation associated with chronic erosive gastritis<sup>[13,14]</sup>. However, to our knowledge, no reports on the use of rebamipide for the treatment of ESD-induced ulcers have been published.

In the present study, we have prospectively evaluated the efficacy of rebamipide monotherapy in comparison to PPI monotherapy for the treatment of iatrogenic ulcers resulting from ESD for early gastric cancers.

## MATERIALS AND METHODS

### Patients

We examined 90 consecutive patients with early gastric cancer who had been treated with ESD at Kinki University Hospital between February 2011 and January 2013.

The study protocol was approved by the Kinki University Ethics Committee, and all participants provided written informed consent before undergoing ESD. In addition, the study was registered at the University Hospital Medical Information Network 000005134. All patients with early gastric cancer, including well-differentiated or moderately differentiated adenocarcinoma, were included in the study. The exclusion criteria were as follows: (1) current use of other anti-ulcer drugs, aspirin, NSAIDs, or prednisolone; (2) treatment with anti-coagulative agents; or (3) previous endoscopic treatment or surgery.

### ESD procedure

ESD was performed with an insulation-tipped knife (KD-610L; Olympus Medical Systems, Tokyo, Japan) and a flush knife (BTDK2618JB; Fujifilm Medical System, Tokyo, Japan). The electrosurgical unit used was A VIO-300D (ERBE). The injection solutions contained glycerin with 1% indigo carmine dye and, depending on the tumor location, hyaluronic acid sodium (0.4%) was also added. The ulcers that developed after ESD were carefully examined endoscopically, and any visible vessels were heat-coagulated by using hot biopsy forceps (KD-410LR; Olympus Medical Systems). Thereafter, the resected specimens were stretched, pinned flat on a rubber plate, and measured.

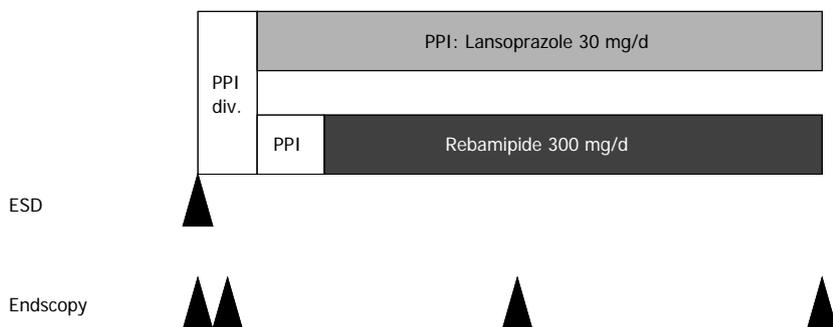
### Study design

The design of this single-center, open-label, prospective, randomized, parallel-controlled study is illustrated in Figure 1.

After ESD, all patients were administered an intravenous infusion of lansoprazole (20 mg; Takepron; Takeda Pharmaceutical, Osaka, Japan) every 12 h for 2 d, and received oral lansoprazole (30 mg/d) for 5 d. On post-operative day 7, the patients were randomly assigned to 2 groups and received either lansoprazole at a dose of 30 mg/d orally (PPI group;  $n = 45$ ), or rebamipide (Mucosta; Otsuka Pharmaceutical Co., Tokyo, Japan) at a dose of 300 mg orally, 3 times a day ( $n = 45$ ) for 8 wk. The primary endpoint was endoscopically documented ulcer healing; complete healing was defined as regression to the S-stage on the Sakita and Miwa scale<sup>[23]</sup>. Moreover, we evaluated the healing rates of atrophic gastritis based on the Kimura and Takemoto classification<sup>[24]</sup>, in the presence or absence of *Helicobacter pylori* (*H. pylori*) infection. We also compared the response of ulcers in relation to their locations in the stomach (lower, middle, or upper).

The secondary endpoint was the ulcer reduction ratio, which was compared according to the ulcer location. For calculation of the ratio, we determined the maximum diameters of the ulcers and the diameters perpendicular to the maximum diameters, which were measured using a bendable endoscopic measuring device (M2-3; Olympus Corp., Tokyo, Japan). Moreover, we determined the ulcer size (maximal diameter  $\times$  diameter perpendicular to the maximal diameter). At 4 and 8 wk after ESD, the healing and reduction rates for the ulcers were compared between the 2 groups. In addition, at 8 wk after ESD, we

POD 0 1 2 7 2 56/d  
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**Figure 1 Study design.** After ESD, all patients were administered an intravenous infusion of lansoprazole (20 mg; Takepron; Takeda Pharmaceutical, Osaka, Japan) every 12 h for 2 d, and received oral lansoprazole (30 mg/d) for 5 d. On postoperative day 7, the patients were randomly assigned to 2 groups and received either lansoprazole at a dose of 30 mg/d orally (PPI group;  $n = 45$ ), or rebamipide (Mucosta; Otsuka Pharmaceutical Co., Tokyo, Japan) at a dose of 300 mg orally, 3 times a day ( $n = 45$ ) for 8 wk. ESD: Endoscopic submucosal dissection; PPIs: Proton-pump inhibitors; div.: Drip intravenous infusion; POD: Postoperative days.

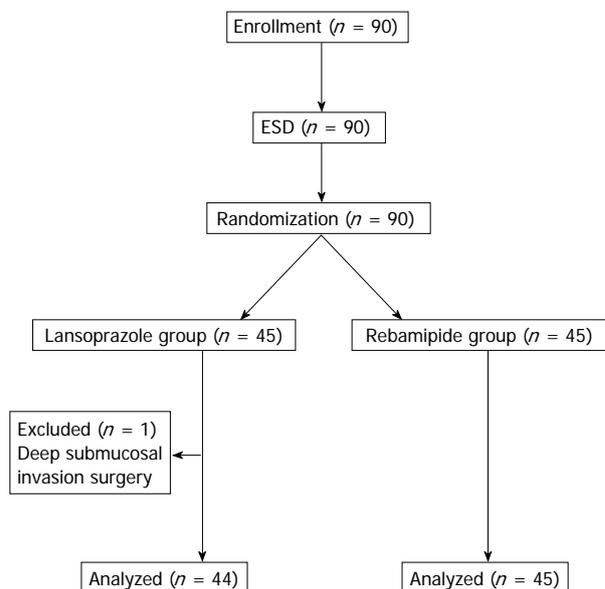
Table 1 Patient characteristics			
	Lansoprazole group ( $n = 45$ )	Rebamipide group ( $n = 45$ )	<i>P</i> -value
Age (yr) (mean ± SD, median)	70 ± 7.8, 72	67 ± 8.0, 67	0.0930
Gender (M/F)	36/9	31/14	0.2269
<i>H. pylori</i> -infection	86.7%	84.4%	0.7643
Smoker	31.1%	33.3%	0.8215
Drinking alcohol	46.7%	42.2%	0.6714
History of disease	46.7%	31.1%	0.1301
Complicated disease	71.1%	71.1%	1.0000
Location of tumors			0.1620
Low	23	16	
Middle	17	26	
Upper	5	3	
Tumor size			0.4986
> 20 (mm)	13	16	
≤ 20 (mm)	32	29	
Histologic classification			0.6939
Tub1	41	42	
Tub2	4	3	
Dissected size (mean ± SD, mm)	30.5 ± 7.8	30.6 ± 6.4	0.9413
Dissected area (mean ± SD, mm <sup>2</sup> )	687.9 ± 393.1.7	712.3 ± 298.6	0.7417
Glandular atrophy			0.3167
C-1	0	0	
C-2	3	2	
C-3	11	14	
O-1	19	13	
O-2	12	13	
O-3	0	3	
CandO			0.6547
C	14	16	
O	31	29	
Intestinal metaplasia	71.1%	68.9%	0.8181

*H. pylori*: *Helicobacter pylori*; M/F: Male/female.

evaluated the scar status of the ESD-induced ulcers according to the Quality of Ulcer Healing (QOUH).

**Statistical analysis**

Patient baseline characteristics and ulcer reduction ratios

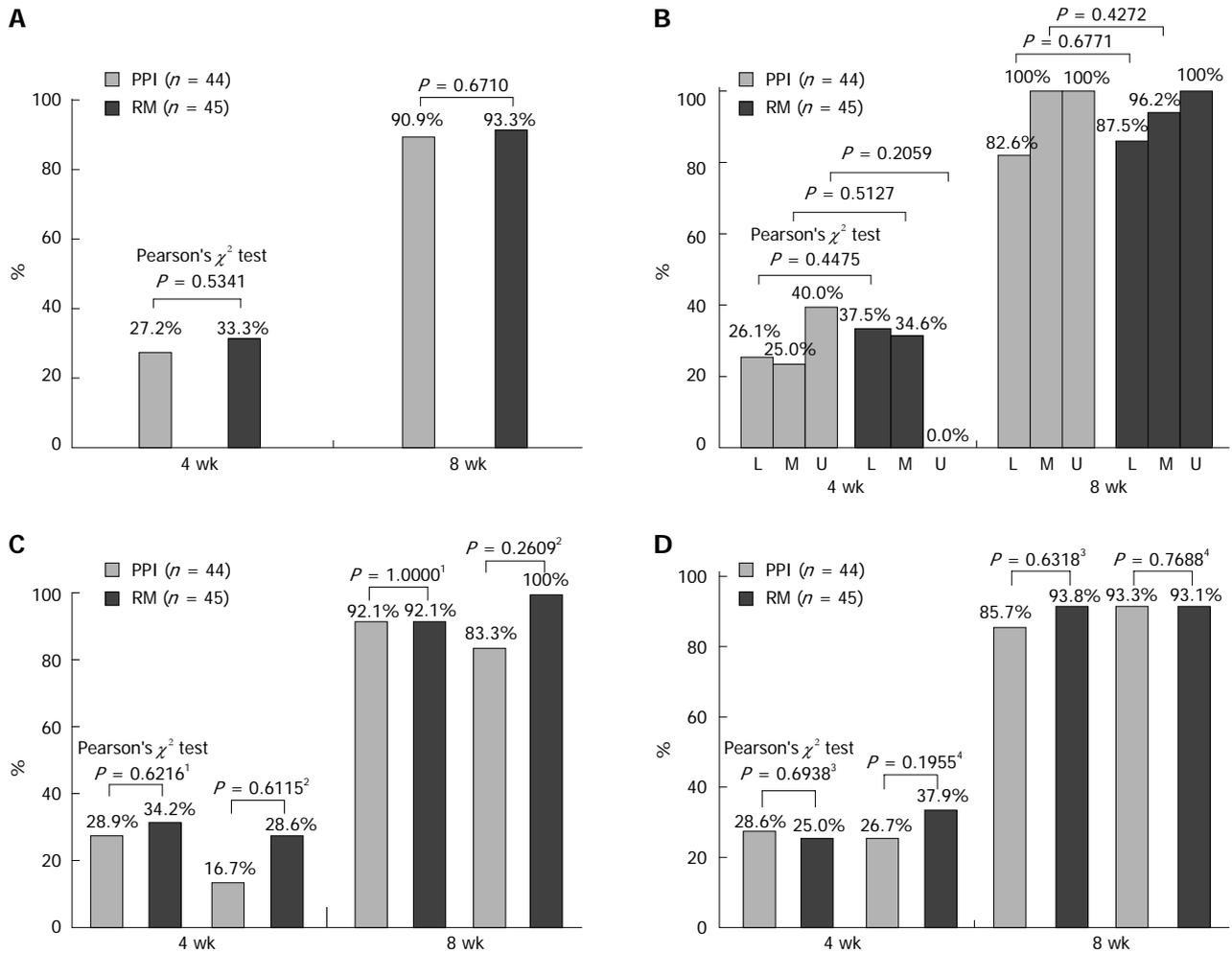


**Figure 2 Flow chart of study participants.** ESD: Endoscopic submucosal dissection.

were compared using Pearson’s  $\chi^2$  test or Student’s *t*-test. Pearson’s  $\chi^2$  test was also used to compare the healing rates of the ESD-induced ulcers and for the evaluation of the scar status of the ESD-induced ulcers according to the QOUH. Statistical significance was defined as  $P < 0.05$ .

**RESULTS**

Table 1 shows the patient characteristics of the 2 treatment groups. No significant differences were noted between the groups with regard to age; gender; tumor location; tumor size; histologic classification; ESD-induced ulcer size; glandular atrophy; history of disease; and the rates of *H. pylori* infection, smoking, drinking alcohol, or the presence of complicated disease or intestinal metaplasia. One patient was excluded from the PPI group because histologic examination of the resected specimen



**Figure 3** Rates of healing in both groups. A: The rates of healing to S stage in both groups; B: The rates of healing to S stage in both groups according to resected location. L: Low stomach, M: Middle stomach; U: Upper stomach. C: The rates of healing to S stage in both groups. <sup>1</sup>*Helicobacter pylori* (*H. pylori*) positive <sup>2</sup>*H. pylori* negative. D: The rates of healing to S stage in both groups in atrophic gastritis. <sup>3</sup>Closed type; <sup>4</sup>Open type. RM: Rebamipide.

indicated deep submucosal invasion (depth ≥ 500 μm; SM2 invasion). Hence, 44 patients in the PPI group and 45 in the rebamipide group constituted the final study cohort (Figure 2).

**Ulcer responses**

The rates of ulcer healing (regression to S-stage) were not significantly different between the PPI group (27.2%) and the rebamipide group (33.3%) at 4 wk ( $P = 0.5341$ ) or at 8 wk (90.9% for the PPI group and 93.3% for the rebamipide group;  $P = 0.6710$ ) (Figure 3A). Moreover, at 4 and 8 wk, the healing rates were not significantly different between the treatment groups with regard to ulcer location (low, middle, or upper stomach; Figure 3B) or with regard to the presence of absence of *H. pylori* infection (Figure 3C). In addition, at 4 and 8 wk, the healing rates of atrophic gastritis (closed or open type) were similar in the 2 treatment groups (Figure 3D).

**Reduction ratios of ESD-induced ulcers**

The reduction ratios of ESD-induced ulcers were similar at 4 and 8 wk in the rebamipide group (98.0% and 99.9%,

respectively) and in the PPI group (97.2% and 99.9%, respectively) (Figure 4). These ratios were not influenced by the locations of the ulcers in the stomach (low, middle, and upper).

**Quality of ulcer healing and adverse events**

Six patients in the PPI group developed unusual gastric lesions, which comprised an overgrowth of granulation tissue at the ulcer site. At 8 wk, the proportion of patients who developed a flat scar in the rebamipide group (100%) was found to be significantly higher than that in the PPI group (86.3%;  $P = 0.0103$ ) (Figure 5). No ulcer bleeding or complications related to the drugs used after ESD were observed in any of the study subjects.

**DISCUSSION**

Some authors have reported that the combination therapy involving PPI and rebamipide is superior to PPI monotherapy in the healing of ESD-induced ulcers<sup>[2-5]</sup>; however, to our knowledge, no reports on the efficacy of rebamipide for the treatment of ESD-induced ulcers

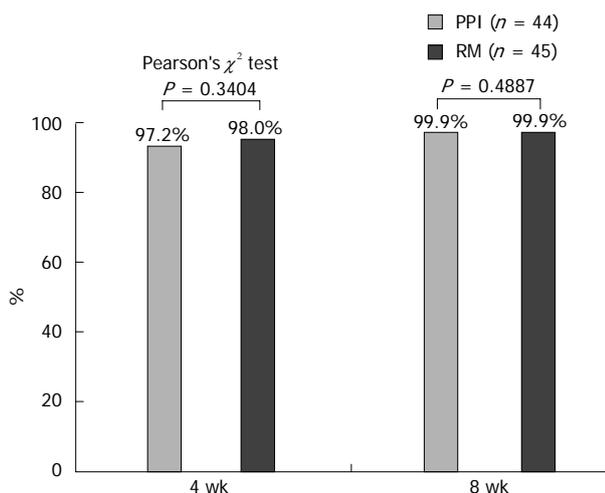


Figure 4 Reduction ratio of the endoscopic submucosal dissection-induced ulcers in both groups. PPI: Proton-pump inhibitor; RM: Rebamipide.

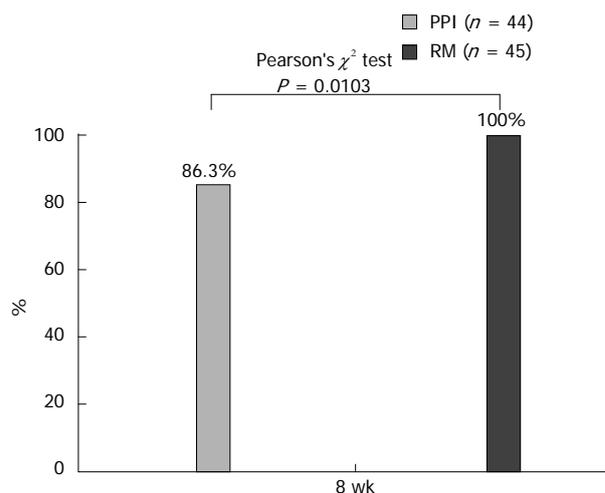


Figure 5 Proportion of patients who developed a flat scar after ulcer healing in both groups. PPI: Proton-pump inhibitor; RM: Rebamipide.

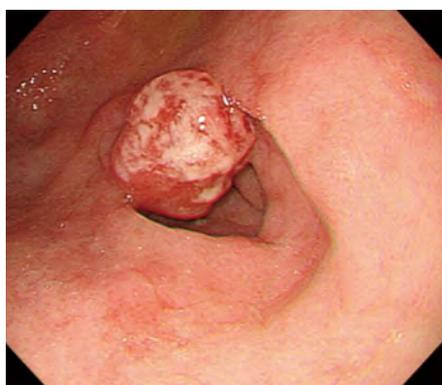


Figure 6 At 8 wk, granulation lesions following ulcer healing in the proton-pump inhibitors treated group.

have been published. In this prospective randomized, parallel-controlled study, we demonstrated that rebamipide monotherapy was as effective as PPI in the healing of ESD-induced ulcers, regardless of the location of the resected cancer, the degree of atrophic gastritis, or the presence of *H. pylori* infection.

Although the response of post-ESD ulcers to PPIs and rebamipide may be similar, the mechanisms of action of these drugs are different. PPIs decrease gastric acid production, whereas rebamipide stimulates the production of prostaglandins<sup>[19]</sup>, epidermal growth factor<sup>[12,20]</sup>, and nitric oxide<sup>[21]</sup>, and decreases the level of oxygen-free radicals<sup>[22]</sup>. These mucosal protective actions of rebamipide appear to promote ulcer healing. Fujiwara *et al*<sup>[3]</sup> showed that 8 wk of PPI and rebamipide treatment was particularly effective for patients with severe atrophic gastritis, classified as O-3. Severe atrophic gastritis may result in the formation of a low-acid environment in the stomach; therefore, acid-suppressive agents such as PPI alone may have a limited effect. However, rebamipide can be effective in this environment because of its different mechanism of action. We believe that this is a contributing factor to the similar efficacies ob-

served between PPIs and rebamipide regardless of the degree of atrophic gastritis.

Previous studies have reported that various mechanisms are involved in the effects of rebamipide on *H. pylori*-positive atrophic gastritis; these include prevention of adhesion of the bacteria to gastric epithelial cells, and inhibition of *H. pylori*-induced secretion of prostaglandin E2 from neutrophils and interleukin-8 expression in gastric epithelial cells<sup>[25-28]</sup>. Terano *et al*<sup>[15]</sup> indicated that the treatment of gastric ulcers with rebamipide promotes ulcer healing regardless of the success or failure of *H. pylori* eradication therapy, and Higuchi *et al* showed that rebamipide prevents the recurrence of gastric ulcers without affecting the *H. pylori* infection status<sup>[10]</sup>. In the present study, PPI and rebamipide appeared to aid in ulcer healing without affecting the *H. pylori* infection status.

Moreover, we noted that the proportion of patients who developed a flat scar at the ulcer site was significantly higher in the rebamipide group than in the PPI group. Thus, rebamipide appears to be more effective than PPIs in improving the QOUH. In animal studies, rebamipide was found to improve the QOUH by increasing the level of prostaglandin E2 and decreasing the levels of malondialdehyde and interleukin-8 in the gastric mucosa<sup>[16]</sup>. In the present study, the unusual elevated gastric lesions that were observed following ulcer healing could not be easily characterized as benign granulation tissue or a malignant recurrence without performing a biopsy (Figure 6). Therefore, we believe that improvement in QOUH is essential for preventing the occurrence of mucosal protrusion due to the growth of granulation tissue.

The most frequent complication that occurs after endoscopic therapy is bleeding, and the rate of intraoperative bleeding is significantly higher with ESD than with EMR. Jeong *et al*<sup>[17]</sup> reported that PPIs may be more effective than histamine H<sub>2</sub> inhibitors in preventing bleeding after ESD by promoting a more rapid healing of these large iatrogenic ulcers<sup>[17]</sup>. Moreover, Uedo *et al*<sup>[18]</sup> indicated that therapy with PPI was more effective than treatment

with histamine H<sub>2</sub> inhibitors in preventing delayed bleeding from ulcers induced by ESD. However, in the present study, no post-ESD bleeding or complications related to the drugs used were noted in the patients receiving rebamipide or PPI treatment; moreover, the ratio of ulcer reduction was at least 90% in both groups at 8 wk after initiation of therapy. Thus, our findings indicated that the rate of intraoperative bleeding was not significantly different between both the groups.

In addition, in the present study, we found that treatment with rebamipide was more cost-effective than treatment with the PPI lansoprazole. The cost of the 56-d treatment course was 4889 yen for rebamipide and 10945 yen for lansoprazole, which is a difference of 44.7%. This high difference in cost may be a factor in determining which medication to prescribe in the treatment of ESD-induced ulcers.

In conclusion, rebamipide monotherapy was equivalent to treatment with a PPI (lansoprazole) in the healing of ulcers induced by ESD for early gastric cancer. The similarity in the treatment efficacy was observed irrespective of the presence of *H. pylori* infection, the severity of atrophic gastritis, or the locations of the ulcers in the stomach. However, rebamipide therapy also resulted in a more favorable QOUL compared with that obtained by PPI treatment. Moreover, the treatment involving rebamipide was more cost-effective compared to the treatment with the PPI lansoprazole for the treatment of ESD-induced ulcers.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Endoscopic submucosal dissection (ESD) is useful for treating early gastric cancer. The artificial ulcer that is generated after ESD is large, and needs a considerably longer healing time compared with conventional endoscopic mucosal resection (EMR). Rebamipide is one of the mucoprotective antiulcer drug, and is widely employed treatment of gastric ulcer in Japan.

### Research frontiers

Previous studies have shown that the combination therapy involving proton pump inhibitor (PPI) and rebamipide is superior to PPI monotherapy in the healing of ESD-induced ulcers; however, to people knowledge, no reports on the efficacy of rebamipide for the treatment of ESD-induced ulcers have been published. Therefore, the authors prospectively investigated differences in healing of ESD-induced ulcers according to treatment with PPI or rebamipide only.

### Innovations and breakthroughs

In this prospective randomized, parallel-controlled study, the authors demonstrated that rebamipide monotherapy was as effective as PPI in the healing of endoscopic submucosal dissection-induced ulcers. In addition, rebamipide treatment was more cost-effective and resulted in a better quality of ulcer healing compared to the PPI lansoprazole treatment.

### Applications

This article suggests that the healing rate of ESD-induced ulcers was similar with rebamipide or PPI treatment; however, rebamipide treatment is more cost-effective and prevents granulation lesions following ulcer healing. However, it is a small study, therefore, a prospective multicenter study with a large sample size should be performed to assess the efficacy of treatment with rebamipide

for endoscopic submucosal dissection-induced ulcers.

## Terminology

ESD is a well-established curative treatment for early gastric cancer. ESD facilitates the collection of larger specimens, regardless of lesion size or location, resulting in a higher rate of en bloc and histologically complete resection. However, the iatrogenic ulcer that develops as a result of ESD is large, and requires a considerably longer healing time compared to that resulting from conventional EMR. Rebamipide is a novel mucosal-protective and ulcer-healing drug that has been widely prescribed in East Asia. Rebamipide stimulates the production of prostaglandins, epidermal growth factor, and nitric oxide, and decreases the level of oxygen-free radicals. These mucosal protective actions of rebamipide appear to promote ulcer healing.

## Peer review

This paper is well written. The clinical results are appropriately described. The authors present the Efficacy of treatment with rebamipide for endoscopic submucosal dissection-induced ulcers. The data indicate the healing rate of ESD-induced ulcers was similar with rebamipide or PPI treatment; however, rebamipide treatment is more cost-effective and prevents granulation lesions following ulcer healing.

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**L- Editor** Cant MR **E- Editor** Zhang DN



## Comparison of pancreatic acinar cell carcinoma and adenocarcinoma using multidetector-row computed tomography

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### Abstract

**AIM:** To distinguish acinar cell carcinoma (ACC) from pancreatic adenocarcinoma (AC) by comparing their computed tomography findings.

**METHODS:** Patients with ACC and AC were identified on the basis of results obtained using surgically resected pancreatotomy specimens. The preoperative computer tomographic images of 6 acinar cell carcinoma patients and 67 pancreatic adenocarcinoma patients in 4 phases (non-contrast, arterial, portal venous, and delayed phase) were compared. The scan delay times were 40, 70, and 120 s for each contrast-enhanced phase. The visual pattern, tomographic attenuation value, and time attenuation curve were assessed and compared between AC and ACC cases using the  $\chi^2$  test, Wilcoxon signed-rank test, and Mann Whitney *U* test.

**RESULTS:** The adenocarcinomas tended to be hypodense in all 4 phases. The acinar cell carcinomas also tended to be hypodense in the 3 contrast-enhanced

phases, although their computed tomographic attenuation values were higher. Further, 5 of the 6 acinar cell carcinomas (83%) were isodense in the non-contrast phase. The time attenuation curve of the adenocarcinomas showed a gradual increase through the 4 phases, and all adenocarcinomas showed peak enhancement during the delayed phase. The time attenuation curve of the acinar cell carcinomas showed peak enhancement during the portal venous phase in 4 cases and during the arterial phase in 2 cases. None of the 6 acinar cell carcinomas showed peak enhancement during the delayed phase.

**CONCLUSION:** The tumor density in the non-contrast phase and time attenuation curve pattern clearly differ between acinar cell carcinomas and adenocarcinomas, and multidetector-row computed tomography can thus distinguish these tumors.

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**Key words:** Pancreatic acinar cell carcinoma; Pancreatic adenocarcinoma; Multidetector-row computed tomography; Visual pattern; Time attenuation curve

**Core tip:** The tumor density in the non-contrast phase and time attenuation curve pattern clearly differ between acinar cell carcinoma and adenocarcinomas, although both tumors tend to be hypodense in the contrast-enhanced phases.

Sumiyoshi T, Shima Y, Okabayashi T, Kozuki A, Nakamura T. Comparison of pancreatic acinar cell carcinoma and adenocarcinoma using multidetector-row computed tomography. *World J Gastroenterol* 2013; 19(34): 5713-5719 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5713.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5713>

## INTRODUCTION

Acinar cell carcinoma (ACC) is a rare malignant epithelial neoplasm that exhibits exocrine enzyme production, and it accounts for approximately 1% of all pancreatic neoplasms<sup>[1]</sup>. ACCs have been reported to be bulky tumors that mainly occur in the pancreatic head<sup>[2]</sup>, and recent reports have shown that ACCs are often accompanied by intratumoral necrosis and have various specific extraparenchymal progression patterns, such as intraductal tumor growth (ITG) and venous tumor thrombus (VTT)<sup>[3-9]</sup>. Several reports have described the computed tomography (CT) findings of ACC: it is typically solitary and is accompanied by an intratumoral hypodense area when large. In terms of the visual pattern, although a few hyperdense ACCs have been reported, most ACCs have been reported to be hypodense on contrast-enhanced CT<sup>[10,11]</sup>. Despite these previous reports on imaging findings, the correct preoperative diagnosis of ACC remains difficult, and ACC is often misdiagnosed as another hypodense pancreatic tumor, namely, adenocarcinoma (AC)<sup>[11]</sup>.

ACCs had been previously considered equally aggressive as ACs<sup>[12,13]</sup>, and pretreatment differentiation between ACC and AC was not considered important. However, in recent years, increasing evidence has shown that ACCs are characterized by less aggressive growth and that ACC shows significantly better long-term survival than AC<sup>[12]</sup>. Further, although no consensus has been reached on surgery for metastatic ACCs, a few reports have described a good prognosis after resection of limited metastatic disease. Because the malignant potential of ACC and AC is significantly different, correct pretreatment distinction between these two tumors is very important.

This study aims to elucidate the characteristic CT findings of ACC to allow accurate diagnosis of even small ACCs. The visual pattern, CT attenuation value, and time attenuation curve (TAC) pattern of ACCs on 4-phase multidetector-row computed tomography (MDCT) were retrospectively reviewed, and the results were compared with those of ACs.

## MATERIALS AND METHODS

### Patients

The study design was approved by the institutional review board. Informed consent was not required because the review of the patients' data was anonymous. After a thorough search of the computerized database of the Hepatobiliary Pancreatic Surgery Division from April 2006 to March 2011, 6 patients with ACC and 88 patients with AC were identified on the basis of results obtained using surgically resected pancreatotomy specimens. Twenty-one AC patients were excluded because CT attenuation values for these tumors could not be measured accurately for the following reasons: halation of indwelling biliary drainage tube (11 ACs), small size and unclear tumor margin (9 ACs), and allergy to contrast media (1 AC). MDCT images of the 6 ACC patients and the remaining

67 AC patients were comparatively reviewed.

### MDCT examination

All MDCT studies were performed using a scanner with 16 rows of detectors (Aquilion 16; Toshiba Medical Systems, Tokyo, Japan). CT images, both unenhanced and contrast enhanced, were routinely obtained with the patient in the supine position during full inspiration. For contrast-enhanced imaging, 100 mL of nonionic contrast material with iodine was administered at a rate of 3.2 cc/s using a mechanical power injector through a 20-gauge angiographic catheter inserted into a forearm vein. The scan delay time was 40 s for the arterial phase, 70 s for the portal venous phase, and 120 s for the delayed phase. Four-phase images (1 unenhanced image and 3 contrast-enhanced images) were routinely obtained. The scanning parameters for each phase were 1-mm collimation, 3-mm slice thickness, 3-mm reconstruction interval, 120 kV, and auto mA.

### Imaging analysis

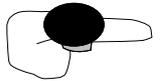
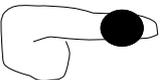
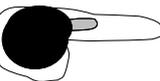
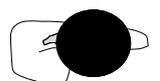
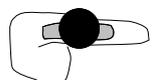
MDCT images were available from the picture archiving and communication system (PACS), and all images were reviewed on the PACS monitor. All CT images were evaluated retrospectively by 2 experienced hepatobiliary and pancreatic surgeons with 13 and 24 years of experience, respectively. CT images were assessed for the visual pattern and CT attenuation value of the ACs and ACCs. The visual pattern of each lesion was classified as hyperdense, isodense, or hypodense, compared to the surrounding normal pancreatic parenchyma in each phase. The CT attenuation value in Hounsfield units was obtained using region of interest (ROI) analysis. To reduce the effect of tumor heterogeneity, one ROI of the largest possible area was identified within the tumor at the level of maximum tumor diameter. The ROI value was calculated as the CT attenuation value of the tumor. Three ROIs of diameter 1 cm were also identified in the normal parenchyma adjacent to the tumor, and the mean of the 3 ROI values was calculated as the CT attenuation value of the surrounding parenchyma. While defining ROIs, special attention was paid to exclude cystic areas, calcification, the pancreatic duct, and the surrounding vessels. TAC patterns were drawn on the basis of each CT attenuation value, and they were compared between the ACCs and ACs.

### Pathological examination and analysis

All the ACCs and ACs in this study were surgically resected, and 2 pathologists reviewed the gross appearance of the tumor specimens and hematoxylin-eosin-stained specimens on microscopic slides. For the ACCs, immunohistochemical analysis was performed for chromogranin and synaptophysin to exclude mixed acinar-endocrine carcinomas (MAEs).

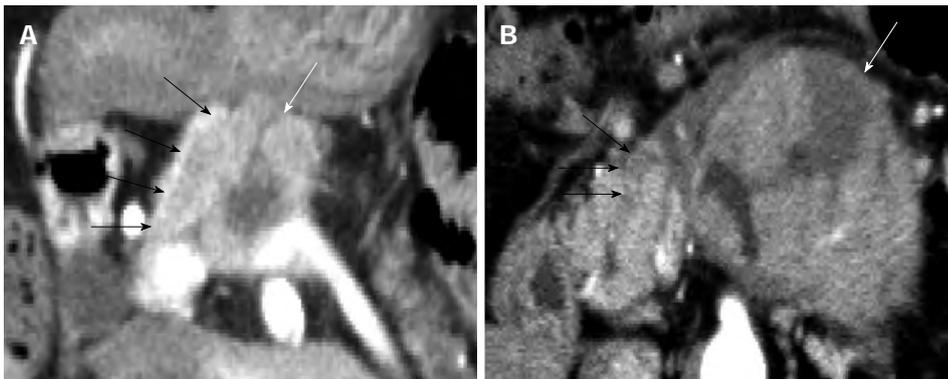
### Statistical analysis

The visual patterns of the ACCs and the ACs were compared using the  $\chi^2$  test. The CT attenuation values were

No.	Age, sex	Pre diag	Scheme	Location/size/surgery	Intra-tumoral necrosis	Intraductal tumor growth	Venous tumor thrombus
1	68, M	AC		Ph/35 mm/PD	<input type="radio"/>		
2	67, M	AC		Pb/48 mm/DP	<input type="radio"/>		<input type="radio"/>
3	77, M	AC		Pt/31 mm/DP	<input type="radio"/>		
4	61, M	AC		Phb/48 mm/PD		<input type="radio"/>	
5	52, M	AC		Pb/87 mm/DP	<input type="radio"/>	<input type="radio"/>	
6	89, M	ACC		Pb/32 mm/DP	<input type="radio"/>	<input type="radio"/>	

Primary tumor       Intraductal tumor growth or venous tumor thrombus       Histologically proven tumor-related findings

**Figure 1 Clinicopathological findings of the acinar cell carcinomas.** M: Male; Pre Diag: Preoperative diagnosis; AC: Adenocarcinoma; ACC: Acinar cell carcinoma; Ph/b/t: Pancreatic head/body/tail; PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy.



**Figure 2 Acinar cell carcinomas with intraductal tumor growth.** A: Case 6, computed tomography (CT) showed the primary acinar cell carcinoma (ACC) in the pancreatic body (white arrow) and the easily recognizable widespread intraductal tumor growth (ITG) (black arrows); B: Case 5, CT shows the primary ACC in the pancreatic body (white arrow) and the small almost-unrecognizable ITG (black arrows).

compared between each phase by using the Wilcoxon signed-rank test. The CT attenuation values of the ACCs and the ACs were compared by using the Mann-Whitney *U* test. Data were analyzed using IBM SPSS Statics 19, and *P* values less than 0.05 were considered statistically significant.

## RESULTS

### Clinicopathological findings

Each pancreatic tumor had been preoperatively diagnosed on the basis of blood examination, CT images and endoscopic findings at weekly hepatobiliary pancreatic conferences involving radiologists, gastroenterologists, and surgeons. Of the ACC cases, 5 tumors had been diagnosed as AC, and only 1 tumor (case 6) had been correctly diagnosed as ACC (Figure 1). In all 6 ACC cases, the patients

were male (mean age, 69 years; range, 52-89 years). Two patients underwent pancreaticoduodenectomy, and the other 4 patients underwent distal pancreatectomy. The maximum diameter of the tumors ranged from 31 to 87 mm, and the mean maximum diameter was 46.8 mm. Five tumors showed intratumoral necrosis. Extraparenchymal tumor extension as ITG and VTT was observed in 3 patients and 1 patient, respectively (Figures 1 and 2). All ACCs were characterized by extensive cellularity and minimal stroma, and the tumor cells showed basophilic cytoplasm and frequently contained eosinophilic granules in the cytoplasm. The tumor cells were arranged in an acinar pattern in 3 ACCs and in a solid pattern in 3 ACCs. Immunohistochemical analysis showed negative reactions for chromogranin and synaptophysin in all cases. Re-examination of the morphological characteristics, cell structure, and immunohistochemical reactions of all resected



**Figure 3** Visual patterns of the adenocarcinoma and the acinar cell carcinoma in the 4 phases. A-D: Adenocarcinoma in the pancreatic tail (circle); The tumor was hypodense in all 4 phases (A: Non-contrast phase; B: Arterial phase; C: Portal venous phase; D: delayed phase). It showed a gradual enhancement pattern across the phases; E-H: Case 1, Acinar cell carcinoma in the pancreatic head (circle); The tumor was isodense and undetectable in the non-contrast phase, although calcification was identified (arrow) (E); It was hypodense in all 3 contrast-enhanced phases (F: Arterial phase; G: Portal venous phase; H: Delayed phase); Contrast enhancement was the strongest in the portal venous phase (G).

**Table 1** Visual pattern of acinar cell carcinoma and adenocarcinoma *n* (%)

	Non-contrast	Arterial	Portal venous	Delayed
ACC	Hypo	Hypo	Hypo	Hypo
6 cases	1 (17)	6 (100)	6 (100)	5 (83)
	Iso			Iso
	5 (83)			1 (17)
AC	Hypo	Hypo	Hypo	Hypo
67 cases	53 (79)	67 (100)	67 (100)	46 (69)
	Iso			Iso
	14 (21)			13 (19)
				Hyper
				8 (12)
<i>P</i> value	<i>P</i> < 0.01	NS	NS	NS

ACC: Acinar cell carcinoma; AC: Adenocarcinoma; Hypo: Hypodense; Iso: Isodense; Hyper: Hyperdense; NS: Not significant.

tumors excluded neuroendocrine tumors and MAEs. All tumors were diagnosed as pure ACCs. Among 67 AC patients, 34 AC patients were male and 33 were female (mean age, 71.4 years; range, 34-87 years). The maximum diameter of the tumors ranged from 12 to 105 mm, and the mean maximum diameter was 35 mm. All tumors were whitish, solid, and associated with dense fibrotic stroma. None of the tumors was accompanied by significant intratumoral necrosis. All tumors were diagnosed as tubular adenocarcinoma; adenocarcinoma variants such as adenosquamous carcinoma, colloid carcinoma, and undifferentiated carcinoma were not observed.

### MDCT findings

**Visual pattern:** Fifty-three ACs (79%) were hypodense while 14 (21%) were isodense in the non-contrast phase

(Figure 3, Table 1). All ACs were hypodense in the arterial and portal venous phases. Forty-six ACs (69%), 13 ACs (19%), and 8 ACs (12%) were hypo-, iso-, and hyperdense in the delayed phase, respectively.

One ACC (17%) was hypodense and 5 (83%) were isodense in the non-contrast phase (Figure 3, Table 1). All ACCs were hypodense in all 3 contrast-enhanced phases, except for 1 tumor, which was isodense in the delayed phase. Thus, the visual pattern was clearly different between ACCs and ACs in the non-contrast phase ( $P < 0.01$ ) (Table 1).

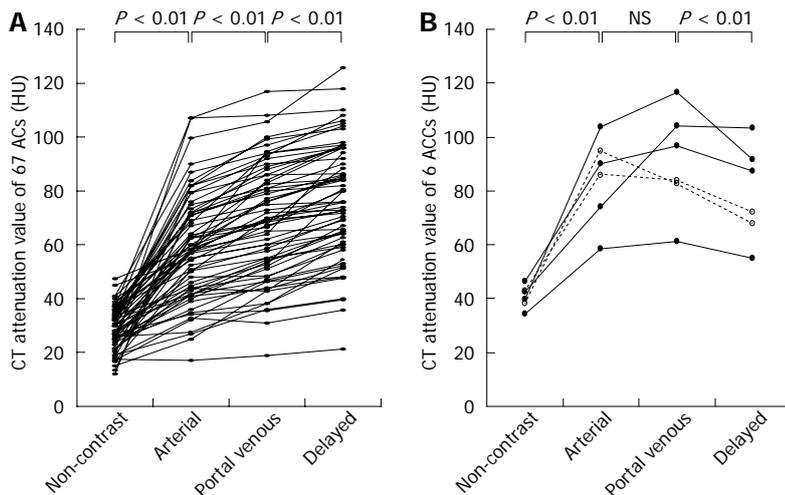
**CT attenuation value and TAC pattern:** The CT attenuation values of the ACs showed a gradually increasing pattern (non-contrast *vs* arterial,  $P < 0.01$ ; arterial *vs* portal venous,  $P < 0.01$ ; portal venous *vs* delayed,  $P < 0.01$ ) (Figure 4). The TAC of all 67 ACs showed peak enhancement during the delayed phase.

The TAC of 4 ACCs showed peak enhancement during the portal venous phase. That of the remaining 2 ACCs showed peak enhancement during the arterial phase, followed by a gradual decline. Unlike the ACs, the ACCs showed significantly higher CT attenuation values in the portal venous phase than in the delayed phase ( $P < 0.01$ ) (Figure 4).

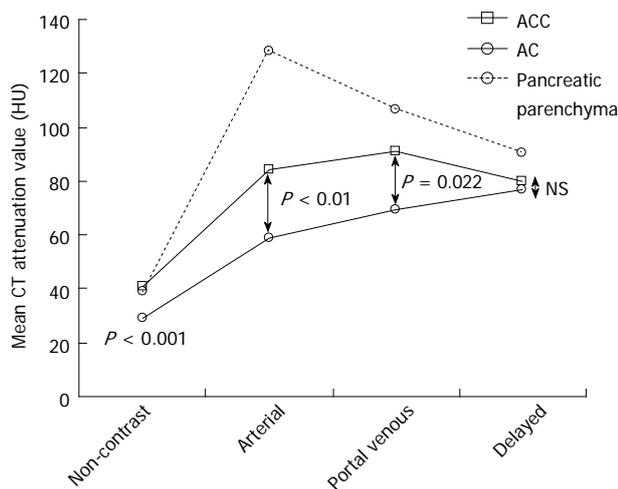
In all 3 phases (non-contrast, arterial, and portal venous), the CT attenuation values of the ACCs were significantly higher than those of the ACs, although the visual patterns of the 2 tumors were clearly different only in the non-contrast phase (Figure 5, Table 1).

## DISCUSSION

Previously, ACCs were considered equally aggressive



**Figure 4** Time attenuation curve of the 67 adenocarcinomas (A) and 6 acinar cell carcinomas (B). Peak enhancement is seen during the delayed phase for all 67 acinar cell carcinomas. Meanwhile, peak enhancement is seen during the portal venous phase for 4 acinar cell carcinomas (ACCs) and during the arterial phase for 2 ACCs. None of the 6 ACCs show peak enhancement during the delayed phase. AC: Adenocarcinomas; CT: Computed tomography; NS: Not significant.



**Figure 5** Mean computed tomography attenuation values of the tumors and the surrounding pancreatic parenchyma in the 4 phases. In 3 phases (non-contrast, arterial, and portal venous phase), the computed tomography (CT) attenuation values of the acinar cell carcinomas (ACCs) were significantly higher than those of the adenocarcinomas (ACs). NS: Not significant.

cancers as ACs<sup>[12,13]</sup>. Therefore, the treatment strategy for both tumors was essentially the same, and preoperative differentiation between ACC and AC was not considered important. However, in recent years, increasing evidence has shown that ACCs exhibit less aggressive growth and significantly better long-term survival than ACs<sup>[12]</sup>. Two recent large population-based studies proved the better prognosis of ACC<sup>[14,15]</sup>. Schmidt *et al.*<sup>[14]</sup> reported the largest ACC series of 865 patients from the National Cancer Database, and they described the 5-year survival rates to be 36.2% and 10.4% for the resected and non-resected cases, respectively. The stage-specific 5-year survival was significantly better for resected ACC than AC (stage I: 52.4% *vs* 28.4%; II: 40.2% *vs* 9.8%; III: 22.8% *vs* 6.8%; IV: 17.2% *vs* 2.8%). These findings suggest that the survival rate is better for ACC than for AC, and even in advanced

ACC cases, survival can be improved by resection. Further, although no consensus has been reached on surgery for metastatic ACCs, a few reports have described a good prognosis after resection of limited metastatic disease. Hartwig *et al.*<sup>[12]</sup> reported that the overall survival did not differ between 9 patients who underwent metastatic disease resection and 6 patients who underwent nonmetastatic disease resection. Suzuki *et al.*<sup>[16]</sup> reported the case of a long-term survivor of metastatic ACC who was successfully treated with repetitive surgery. Because surgery might result in longer survival for ACC patients, even those with metastatic disease, the malignant potential of ACC and AC is thought to be significantly different, and accurate diagnosis of ACC is very important.

Recent reports on CT have shown that ACCs are typically solitary, and they are homogeneously enhanced when the lesion is small but may contain hypodense areas because of necrosis if the lesion is large<sup>[11,17]</sup>. In terms of the visual pattern in contrast-enhanced phases, although a few reports described ACC to be a hyperdense tumor in the arterial phase<sup>[18]</sup>, some reported that it tended to be enhanced less than the adjacent normal pancreatic parenchyma<sup>[10,11]</sup>. Chiou *et al.*<sup>[17]</sup> reported on the CT manifestations of 8 ACCs, of which 6 were hypodense and 2 were isodense in the early arterial and portal venous phases. As shown in previous reports, ACCs tended to be hypodense in all 3 contrast-enhanced phases in the current study, and hypervascular pancreatic tumors, such as neuroendocrine tumor or metastatic renal cell carcinoma, were not included among the preoperative differential diagnoses. Although several such valid imaging findings of ACCs are available, accurate preoperative imaging-based diagnosis of ACCs, especially small ACCs, remains difficult<sup>[19]</sup>. In the current study, ACC was correctly diagnosed on the basis of recognizable widespread ITG on CT images in only 1 case (Figure 2A). Although the characteristic progression patterns of ITG or VTT were observed in 3 other cases (cases 2, 4, and 5), ACC was not preop-

eratively diagnosed in these cases. Because the ITG or VIT lesions were small and continuous with the primary tumor in these cases, they could not be considered to be the tumor that had progressed into the pancreatic duct or splenic vein, and they were regarded as part of the primary tumor (Figure 2B). To identify novel indicators for the accurate diagnosis of ACC, the CT attenuation values of ACC were compared with those of AC, which was the most frequently suspected disease in the preoperative diagnosis in our ACC cases, and we found that ACCs had a unique TAC pattern. The TAC of the ACCs showed the peak enhancement during the portal venous phase in the 4 ACCs, and during the arterial phase in the 2 ACCs. None of the 6 ACCs showed the peak enhancement during the delayed phase. This TAC pattern of ACC was clearly different from that of AC. Several studies have reported the CT findings of pancreatic AC, and it is well known that AC with fibrous stroma appears hypodense with delayed enhancement on dynamic CT<sup>[20-22]</sup>. The ACs in the current study also showed the gradual enhancement pattern, and all 67 ACs showed the peak enhancement during the delayed phase. Although the reasons for the different TAC pattern of ACs and ACCs have not been elucidated, we speculate that the degree of intratumoral fibrosis is one. Hattori *et al.*<sup>[23]</sup> reported that the CT attenuation value of ACs correlated negatively with the extent of intratumoral fibrosis in 3 contrast-enhanced phases. The scanty fibrous stroma in the ACCs might have led to their higher CT attenuation values compared with those of the ACs. The isodensity of most ACCs in the non-contrast phase, which is clearly different from the hypodensity of most ACs, is also thought to reflect the degree of fibrosis. In this study, 3 relatively small ACCs (31, 32, and 35 mm in diameter) also showed the specific TAC pattern. Thus, this TAC pattern might be useful to distinguish ACCs from ACs, especially when they are small and have no distinguishing morphological features. Further, in the future, it may be possible to apply these different patterns of enhancement on MDCT to echoendoscopy.

Echoendoscopy has been reported to be superior to any other modality with respect to spatial resolution, and it can accurately detect small pancreatic lesions<sup>[24-26]</sup>. Contrast-enhanced endoscopic ultrasonography (CE-EUS) has emerged as a recent technological development, and this modality can be used to evaluate the degree of enhancement in pancreatic lesions<sup>[25,26]</sup>. Kitano *et al.*<sup>[26]</sup> reported that CE-EUS was useful for characterizing pancreatic lesions and that it was superior to MDCT for diagnosing small lesions. Although, to our knowledge, no study has compared the enhancement pattern between ACs and ACCs using echoendoscopy, this modality may prove useful for distinguishing these 2 pancreatic tumors.

Despite the novel findings of this study, it does have some limitations. Firstly, the number of ACC cases included is small, and it is not clear whether or not every ACC definitely shows the unique TAC pattern. Another limitation is that the actual effectiveness of this TAC pat-

tern is unclear, because of the retrospective nature of this study. Further investigation is necessary to prove that the TAC pattern is specific to ACCs and that it is actually useful in distinguishing ACCs from other pancreatic tumors.

In conclusion, the tumor density in the non-contrast phase and TAC pattern are clearly different between ACCs and ACs, although both tumors tend to be hypodense in the contrast-enhanced phases.

## COMMENTS

### Background

Acinar cell carcinoma (ACC) is a rare malignant epithelial neoplasm that exhibits exocrine enzyme production, and it accounts for approximately 1% of all pancreatic neoplasms. ACCs have been reported to be bulky tumors that mainly occur in the pancreatic head, and recent reports have shown that ACCs are often accompanied by intratumoral necrosis and have various specific extraparenchymal progression patterns, such as intraductal tumor growth and venous tumor thrombus. Several reports have described the computed tomography (CT) findings of ACC: it is typically solitary and is accompanied by an intratumoral hypodense area when large. In terms of the visual pattern, although a few hyperdense ACCs have been reported, most ACCs have been reported to be hypodense on contrast-enhanced CT.

### Research frontiers

ACCs had been previously considered equally aggressive as ACs, and pretreatment differentiation between ACC and AC was not considered important. However, in recent years, increasing evidence has shown that ACCs are characterized by less aggressive growth and that ACC shows significantly better long-term survival than AC. Further, although no consensus has been reached on surgery for metastatic ACCs, a few reports have described a good prognosis after resection of limited metastatic disease. Because the malignant potential of ACC and AC is significantly different, correct pretreatment distinction between these two tumors is very important.

### Innovations and breakthroughs

The tumor density in the non-contrast phase and time attenuation curve pattern clearly differ between acinar cell carcinomas and adenocarcinomas, and multidetector-row computed tomography can distinguish these tumors.

### Applications

Each pancreatic tumor had been preoperatively diagnosed on the basis of blood examination, CT images and endoscopic findings at weekly hepatobiliary pancreatic conferences involving radiologists, gastroenterologists, and surgeons.

### Peer review

This is an interesting paper. The number of acinar carcinomas is relatively small but still there are some interesting results. Suggest authors edit the paper before publication if they have more cases in hand.

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## Laparoscopic-endoscopic cooperative surgery for gastric submucosal tumors

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### Abstract

**AIM:** To assess the feasibility, safety, and advantages of minimally invasive laparoscopic-endoscopic cooperative surgery (LECS) for gastric submucosal tumors (SMT).

**METHODS:** We retrospectively analyzed 101 consecutive patients, who had undergone partial, proximal, or distal gastrectomy using LECS for gastric SMT at Peking Union Medical College Hospital from June 2006 to April 2013. All patients were followed up by visit or telephone. Clinical data, surgical approach, pathological features such as the size, location, and pathological type of each tumor; and follow-up results were analyzed. The feasibility, safety and effectiveness of LECS for gastric SMT were evaluated, especially for patients with tumors located near the cardia or pylorus.

**RESULTS:** The 101 patients included 43 (42.6%) men

and 58 (57.4%) women, with mean age of  $51.2 \pm 13.1$  years (range, 14-76 years). The most common symptom was belching. Almost all ( $n = 97$ ) patients underwent surgery with preservation of the cardia and pylorus, with the other four patients undergoing proximal or distal gastrectomy. The mean distance from the lesion to the cardia or pylorus was  $3.4 \pm 1.3$  cm, and the minimum distance from the tumor edge to the cardia was 1.5 cm. Tumor pathology included gastrointestinal stromal tumor in 78 patients, leiomyoma in 13, carcinoid tumors in three, ectopic pancreas in three, lipoma in two, glomus tumor in one, and inflammatory pseudotumor in one. Tumor size ranged from 1 to 8.2 cm, with 65 (64.4%) lesions  $< 2$  cm, 32 (31.7%)  $> 2$  cm, and four  $> 5$  cm. Sixty-six lesions (65.3%) were located in the fundus, 21 (20.8%) in the body, 10 (9.9%) in the antrum, three (3.0%) in the cardia, and one (1.0%) in the pylorus. During a median follow-up of 28 mo (range, 1-69 mo), none of these patients experienced recurrence or metastasis. The three patients who underwent proximal gastrectomy experienced symptoms of regurgitation and belching.

**CONCLUSION:** Laparoscopic-endoscopic cooperative surgery is feasible and safe for patients with gastric submucosal tumor. Endoscopic intraoperative localization and support can help preserve the cardia and pylorus during surgery.

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**Key words:** Laparoscopic-endoscopic cooperative surgery; Gastric submucosal tumor; Minimally invasive surgery; Laparoscopy; Endoscopy

**Core tip:** We retrospectively analyzed 101 consecutive patients who had undergone partial, proximal or distal gastrectomy using laparoscopic-endoscopic cooperative surgery (LECS) for gastric submucosal tumor (SMT) at Peking Union Medical College Hospital from June 2006 to April 2013. Ninety-seven patients underwent surgery with preservation of the cardia and

pylorus, with the other four patients undergoing proximal or distal gastrectomy. LECS is feasible and safe for gastric SMT, especially for patients with tumors near the cardia or pylorus. Intraoperative localization and support by endoscopy can help preserve the cardia and pylorus during surgery.

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## INTRODUCTION

Since the first gastrectomy was performed in 1880, surgical methods have developed rapidly due to improvement in anastomosis techniques, surgical staplers, and gastrointestinal tube application<sup>[1]</sup>. Moreover, since the first cholecystectomy by electronic laparoscopy was performed in 1987, minimally invasive laparoscopic surgery has become more popular for its lower postoperative morbidity rates and faster postoperative recovery<sup>[2-5]</sup>. Minimally invasive surgery is suitable for benign gastric lesions, especially for gastrointestinal stromal tumors (GISTs). Although GISTs are potentially malignant, nodal metastasis is rare. Therefore, excision of the tumor with negative margins but without lymphadenectomy has become a standard approach, while GISTs are indicated for minimally invasive partial gastrectomy<sup>[6-9]</sup>.

Although gastric small mucosal tumors (SMT) have been resected laparoscopically, this type of surgery is associated with two potential problems. Laparoscopy may be unable to determine the location of gastric SMTs, because of their small size or intraluminal growth pattern. In addition, complications may arise during the laparoscopic removal of SMTs located near the cardia or pylorus; these complications can include stenosis or damage to the cardia or pylorus. We have therefore developed a technique, called minimally invasive laparoscopic-endoscopic cooperative surgery (LECS), for removal of SMTs. This paper represents our analysis of findings in 101 patients who successfully underwent LECS for gastric SMT at the Department of General Surgery, Peking Union Medical College Hospital, from June 2006 to April 2013.

## MATERIALS AND METHODS

### Clinical data

From June 2006 to April 2013, 101 patients successfully underwent LECS for gastric SMT at the Department of General Surgery, Peking Union Medical College Hospital; the cardia and pylorus were preserved in 97 of these patients. In addition to routine preoperative tests, all patients underwent upper gastrointestinal endoscopy with

endoscopic ultrasound (EUS) and a computed tomography (CT) scan with three-dimensional gastric display. Demographic and clinicopathological characteristics were analyzed retrospectively. Demographic features assessed included patient sex and age, the length of the operation, estimated blood loss, and rate of conversion to open surgery. Postoperative data included time to bowel function recovery (normal passage of gas), surgical complications (*e.g.*, leakage, stenosis, and bleeding), and length of postoperative hospital stay. The clinicopathological characteristics of the SMTs included their size, location, and pathological type.

### Surgical procedures

LECS was performed with the patient under general anesthesia in the reverse Trendelenburg position. The surgeon stood between the patient's legs, the first assistant was to the right or the left of the patient's body, the laparoscopist to the right of the patient's legs, and the gastroscopist to the left of the patient's head.

### Setup for laparoscopic surgery

A camera port was inserted into the inferior (1 cm) umbilical incision (10 mm port) using an open technique. Three additional ports (two 5 mm and one 12 mm in diameter) were inserted into the left upper and right upper quadrants and the inferior xiphoid process (on the right or left side according to the location of the SMT), respectively, under a pneumoperitoneum of 1.60-1.86 kPa, with a laparoscopic view (30° angle range).

### Endoscopic procedures

With the patient anesthetized, the endoscope was inserted through the oropharynx. The mucosae of the esophagus and stomach were viewed, taking care not to infuse too much air into the stomach. The location of the SMT was confirmed, all liquids and gas were withdrawn, and the endoscope was withdrawn through the cardia to remain in the esophagus<sup>[10]</sup>.

### Operative approaches

**Tumors within the anterior wall of the stomach:** The omentum was detached and a little air was allowed to fill the stomach endoscopically. Using both the laparoscope and the endoscope, the location of the SMT was confirmed by the method of touch and marked by one or two suture lines. The gastric wall, including the SMT, was elevated with two seromuscular sutures placed opposite each other and 2-4 cm from the lesion. The tumors, as well as some normal gastric tissues, were removed with a linear endoscopic gastrointestinal stapler (*e.g.*, EC60). If the tumor was located near the esophagogastric junction or pyloric ring, the endoscope was placed distally into the stomach or duodenum to protect the normal gastric tissues from stenosis or damage. After the lesion was resected, direct intraluminal visualization was performed to ensure that the tumor was totally removed and that there was no bleeding or leakage. The amount of air in

**Table 1 Demographic and clinical characteristics of the 101 patients who underwent laparoscopic and endoscopic cooperative surgery for gastric submucosal tumors *n* (%)**

Parameters	Statistics
No. of patients	101
Age (yr)	51.2 ± 13.1 (range 14-76)
Sex	
Male	43 (42.6)
Female	58 (57.4)
Chief complaint	
Dyspepsia (regurgitation, eructation, belching, epigastralgia, and epigastric discomfort)	69 (68.3)
Physical examination (asymptomatic)	27 (26.7)
Melena	5 (5.0)
Tumor location	
Cardia	3 (3.0)
Gastric fundus	66 (65.3)
Gastric body	21 (20.8)
Gastric antrum	10 (9.9)
Pylorus	1 (1.0)
Distance between the tumor and cardia or pylorus (cm)	3.4 ± 1.3 (minimum 1.5)

Data are presented as mean ± SD.

the stomach and peritoneum was balanced, resulting in a good visual field.

#### Tumors within the posterior wall of the stomach:

The proximate curvature was detached to expose the tumor, using, for example, the Ligasure vascular sealing system. The posterior wall was rotated, and the tumor was resected using a technique similar to that described for anterior lesions.

#### Tumors within the lesser curvature (anterior and posterior gastric wall borderline) of the stomach:

The small omentum was detached to expose the tumor, followed by tumor resection using the technique described above. For larger tumors, the left gastric vessels were cut off to prevent both operative and postoperative bleeding. Endoscopic support was especially important for tumors located near the esophagogastric junction<sup>[11]</sup>.

The resected tumor was placed in a specimen retrieval bag located outside the left upper quadrant port. The tumor was cut open along the suture lines, and any ruptures in tumor integrity were assessed. The tumor was measured, immersed in 10% formalin solution, and sectioned. The sections were routinely stained with hematoxylin and eosin, and the number of mitotic figures per 50 high powered fields (HPF) was counted. Risk classifications for GIST were those described by the National Institutes of Health (NIH) in 2008. Gastric GIST was confirmed by immunohistochemistry, using antibodies to identify CD-117 (c-kit), CD-34, and DOG-1.

#### Follow-up

All patients were followed up by visit or telephone after 1, 3, 6, 9, 12, 24, 36, 48, and 60 mo. Each follow-up included a medical history review of any reports of abdominal

discomfort, as well as CT scans and upper gastrointestinal endoscopy to exclude tumor recurrence or metastasis.

#### Statistical analysis

Data are expressed as mean ± SD. All analyses were performed using SPSS 12.0 software (SPSS, Chicago, IL, United States).

## RESULTS

Surgery was successful in all 101 patients. The demographic and clinical characteristics of the 101 patients are depicted in Table 1. Three patients each had two GISTs.

Of the 101 patients, four underwent proximal or distal gastrectomy, including three with tumors located at the cardia, and one with a tumor located at the pylorus. The remaining 97 patients had preservation of the cardia and pylorus. During surgery, tumor location could not be confirmed by laparoscopy alone in 92 patients.

The mean operation time was 113 ± 36 min, and none of these patients required conversion to open surgery. Mean estimated blood loss was 36 ± 18 mL. The postoperative course of all patients was uneventful, with no anastomosis leakage. One patient who underwent proximal gastrectomy had an anastomotic stenosis because of scar physique. This patient was successfully treated by balloon dilatation under X-ray fluoroscopy. One patient experienced anastomotic bleeding and was successfully treated by conservative methods (drug hemostasis and blood transfusion). The average time to first gas passage was 2.9 ± 0.9 d, the average time for nasogastric tube placement was 1.9 ± 0.5 d, and the average postoperative hospital stay was 4.2 ± 1.1 d (Table 2). Seven patients underwent simultaneous laparoscopic cholecystectomy for gallstones, and two underwent simultaneous endoscopic polypus dissection.

All the resected tumors were cut open along the suture lines, with none showing evidence of rupture.

The clinicopathological characteristics of the submucosal stomach tumors, including their location, are shown in Table 3. Of the 101 tumors, 78 (77.2%) were GISTs, with 53 located in the gastric fundus, 14 in the gastric body, seven in the antrum, three in the cardia, and one in the pylorus. The remaining tumors included 13 (12.9%) leiomyomas, 11 in the gastric fundus and two in the gastric body; three (3.0%) ectopic pancreases, two in the gastric fundus and one in the antrum; three (3.0%) carcinoids, two in the gastric body and one in the antrum; two (2.0%) lipomas, one each in the gastric body and antrum; one (1.0%) glomus tumor in the gastric body; and one (1.0%) inflammatory pseudotumor in the gastric body. Maximum tumor size ranged from 1 to 8.2 cm, with 65 (64.4%) lesions < 2 cm in size, 32 (31.7%) > 2 cm, and four > 5 cm.

Gastric GIST was confirmed by immunohistochemistry in 78 patients, with 68 (87.2%) positive for CD117, 65 (82.9%) positive for CD34, and 65 (82.9%) positive for DOG1. Using the NIH biological risk classification for GIST<sup>[12]</sup>, we found that 54 (69.2%) tumors were of

**Table 2** Operative data for laparoscopic and endoscopic cooperative surgery *n* (%)

Parameters	Statistics
Operation time (min)	113 ± 36
Conversion to open surgery	0 (0)
Intraoperative blood loss (mL)	36 ± 18
Postoperative complications	
Gastric fullness	0 (0)
Anastomotic leakage	0 (0)
Anastomotic stenosis	1 (1.0)
Anastomotic bleeding	1 (1.0)
Postoperative hospital stay (d)	4.5 ± 2.1
Time for nasal-gastric tube placement (d)	1.9 ± 0.5
Time until bowel function recovery (d)	2.9 ± 0.9

Data are presented as mean ± SD.

very low risk, including 41 in the gastric fundus, seven in the gastric body, four in the antrum, and two in the cardia; and 16 (23.5%) were of low risk, including eight in the gastric fundus, four in the gastric body, two in the antrum, one in the cardia, and one in the pylorus. Six tumors (7.7%), of mean size  $5.4 \pm 1.3$  cm, were of moderate risk, including three in the gastric fundus, two in the gastric body, and one in the antrum. Two tumors (2.6%) were of high risk, one located in the gastric fundus was 8.2 cm in size; and the second, located in the gastric body, showed 13 mitotic figures/50 HPF. The first patient was treated with imatinib for 2 mo before the surgery, which decreased the tumor size from 8.8 to 8.2 cm in diameter. The eight patients in the moderate- and high-risk classes were treated with adjuvant imatinib for 1-2 years.

All the patients were followed up after LECS, for a mean time of 28 mo (range, 1-69 mo). The three patients who underwent proximal gastrectomy developed symptoms of regurgitation, eructation, and belching. None of the 101 patients who underwent LECS showed evidence of tumor recurrence, metastasis, nutritional disturbances (*e.g.*, weight loss, vitamin deficiency, deficiency of trace elements), or decreased quality of life. One patient developed primary liver cancer 2 years and 4 mo after LECS, but this patient remains alive. In addition, none of the patients with preserved cardia and pylorus experienced any symptoms of epigastric discomfort.

## DISCUSSION

We have shown here that LECS is feasible, yielding satisfactory surgical results, in patients with gastric SMT. Usually, gastric SMTs are resected by open surgery, either distal or proximal gastrectomy<sup>[13]</sup>. Operation time and postoperative hospital stay are longer, and many patients develop gastroesophageal reflux disease (GERD). Quality of life may decrease, and the risk of remnant gastric cancer or esophageal carcinoma may increase. In contrast, LECS requires a relatively small resection of the healthy gastric wall, with very low rates of postoperative morbidity and mortality. Of our 101 patients, only two experienced postoperative complications, one with anastomotic

**Table 3** Clinicopathologic characteristics of submucosal tumors *n* (%)

Parameters	Statistics
Pathological diagnosis	
Gastrointestinal stromal tumor	78 (77.1)
Leiomyoma	13 (12.9)
Ectopic pancreas	3 (3.0)
Carcinoid	3 (3.0)
Lipoma	2 (2.0)
Glomus tumor	1 (1.0)
Inflammatory pseudotumor	1 (1.0)
Tumor size (cm)	4.9 ± 0.6

Data are presented as mean ± SD.

stenosis and one with anastomotic bleeding. Although tumors with an extragastric growth pattern can be easily treated using conventional laparoscopic wedge resection, laparoscopic methods alone have some limitations for the resection of gastric SMTs. Laparoscopy has been found to be less efficient than open surgery in removing small tumors and tumors located in the posterior gastric wall and lesser curvature of the stomach. In addition, the removal of large tumors and those located near the cardia or pylorus can result in post-operative complications, such as stenosis or damage to the cardia or pylorus.

All of our patients routinely underwent two important preoperative tests, upper gastrointestinal endoscopy with EUS and CT scan with a three-dimensional gastric display, both of which are very important for this surgery. EUS was used to assess depth of tumor invasion, lesion location, tumor size, and growth pattern<sup>[14-18]</sup>. The diagnostic accuracy of EUS, however, may be affected by technical problems or skills or the subjective view of the operator, whereas the diagnostic accuracy of CT scanning was less subjective. CT three-dimensional imaging was helpful in assessing tumor size, the distance between the tumor and local tissues (cardia and pylorus), and the diagnosis and staging of SMTs. Use of these two tests could therefore determine whether localized gastric SMTs can be resected.

Endoscopic submucosal dissection (ESD) performed by experienced endoscopists has been used to remove gastric SMTs<sup>[19,22]</sup>. We found that 78 of our 101 (77.2%) SMTs were GISTs. GISTs are a type of mesenchymal neoplasm, originating from Cajal cells; are located in the submucous, muscularis propria, or subserous layer; and have an intraluminal or extrinsic growth pattern. ESD resection of tumors in the muscularis propria, while preserving the integrity of the serous layer, is very difficult. ESD alone may result in high rates of resection failure, intraoperative bleeding, and perforation. In addition, this procedure cannot easily differentiate between benign and malignant tumors. Since GISTs are regarded as potentially malignant and in need of complete resection, ESD alone should not be used to remove gastric SMTs.

The development of the LECS procedure has expanded the range of minimally invasive surgery. The endoscopic assistant cut the exact edges from the gastric

lumen, followed by tumor resection aided by endoscopy. Endoscopic support could reduce complications, such as stenosis or damage to the cardia or pylorus, especially when the tumor is located in the gastric fundus or antrum. Moreover, direct intraluminal visualization can confirm that the tumor has been totally removed, that there is no bleeding from the suture lines, and that there are no perforations. When observing through the endoscope, the pneumoperitoneum should be at lower pressure and the laparoscope should be removed for a better view. All gas and liquid should be removed endoscopically for better laparoscopic procedures. Laparoscopy may be sufficient, however, for large tumors, for tumors located near the cardia and pylorus, and for tumors with an extrinsic growth pattern. Even in these situations, however, endoscopic support is important for protecting the cardia and/or pylorus from damage during resection, even if the endoscope is not need to confirm tumor location. LECS can therefore improve the success rates and outcomes of minimally invasive surgery without postoperative morbidity or mortality.

The sphincter muscles in the cardia and pylorus are important anatomical structures for preserving regurgitation. Although 59.1% of SMTs were reported located at the fundus<sup>[11]</sup>, we found that the percentage was higher, 67.9%. Resection of the cardia can cause symptoms like heartburn due to gastric acid regurgitation. These patients may have to take medicines like proton pump inhibitors for a long time, reducing patient quality of life, and may develop GERD or esophageal carcinoma. Of our 101 patients, only three underwent proximal gastrectomy, with all three developing symptoms of regurgitation, eructation, and belching. Similar findings would be observed after resection of the pylorus, since duodenal juice would regurgitate into the remnant stomach, causing inflammation at the suture lines and corresponding symptoms and ultimately leading to remnant gastric cancer<sup>[21,22]</sup>. Therefore, it is very important to preserve these important anatomical structures. LECS can decrease the risk to resect the cardia and pylorus. We found that the minimum distance from the edge of the tumor to the cardia was 1.5 cm. The importance of endoscopic support was inversely correlated with the distance between the tumor edge and the cardia or pylorus<sup>[23]</sup>. In addition, GISTs are supplied by many blood vessels. When resecting larger tumors within the lesser curvature, the left gastric vessels should be cut off to prevent postoperative bleeding. In this study, one 76-year-old patient experienced anastomotic bleeding, because of atherosclerosis. After 2 d of conservative therapy, consisting of blood transfusions, he got better and was discharged.

All 101 of our patients underwent minimally invasive surgery, with LECS in 97 resulting in the preservation of the cardia and pylorus. None of these patients required conversion to open surgery. Intraoperative bleeding was limited and recovery of bowel function was rapid, with a low postoperative morbidity (except for one patient each with anastomotic stenosis and bleeding), and no

postoperative mortality. Postoperative hospital stay was much shorter than in several previous studies. Except for the three patients who underwent proximal gastrectomy, none developed symptoms like GERD and their quality of life did not decrease over a relatively long-term follow-up, suggesting the importance of preserving the anatomical structure and physical function of the cardia and pylorus. None of our 78 patients with gastric GIST developed tumor recurrence or metastasis after LECS, regardless of risk classification, indicating that total resection of SMTs, including potentially malignant GISTs, by the LECS techniques yields satisfactory surgical outcomes. We found that 50% of tumors classified as moderate or high risk, and most with more than five mitoses per 50 HPFs, were located at the gastric fundus. Patients in moderate- and high-risk categories required adjuvant imatinib<sup>[24]</sup>. We found that two patients had tumors < 5 cm, but more than 10 mitotic figures per 50 HPFs.

LECS can be used for two types of partial gastrectomy. The first consists of laparoscopic wedge resection of gastric SMTs and distal or proximal gastrectomy under endoscopic guidance; and the second consists of laparoscopic cutting of the anterior wall of the stomach, to expose SMTs in the posterior gastric wall, followed by partial resection of the posterior gastric wall. All 101 of our patients with SMTs underwent complete resection, even if the tumors were located in the posterior, the lesser curvature of the stomach or near the cardia or pylorus. The greater curvature of the stomach was detached, the stomach was turned axially, and wedge resection was performed. A good view during this procedure requires that the amount of air in the stomach and peritoneum should be balanced.

LECS is indicated for the removal of SMTs (*e.g.*, leiomyomas, lipomas, and schwannomas), polyps with broad stalks, gastric epithelial tumor degeneration (moderate or severe atypical hyperplasia), lesions with low potential for malignancy (*e.g.*, carcinoid tumors and GISTs), and early-stage, localized gastric carcinomas<sup>[25]</sup>. Because GISTs may easily rupture during laparoscopic surgery, resulting in peritoneal seeding, the integrity of a resected GIST is regarded as a significant prognostic factor. Before 2007, the guidelines of the National Comprehensive Cancer Network did not recommend laparoscopic surgery for GIST resection, except for tumors < 2 cm in diameter and with a low risk of rupture. Although almost one-third of the tumors in this study were > 2 cm in diameter, LECS was successful for all tumors, regardless of tumor size. These findings indicate that the performance of laparoscopic and endoscopic techniques by skilled operators, non-contact with the tumor during surgery, and the use of a specimen retrieval bag are key factors for good surgical results. Tumors > 5 cm in diameter require resection of a relatively large portion of healthy stomach to ensure tumor integrity without rupture<sup>[26]</sup>.

This study had several limitations, including its retrospective design and lack of comparisons with open or laparoscopic surgery. Prospective, multicenter, compara-

tive studies are needed to evaluate the role of LECS for gastric SMT.

In conclusion, we have shown here that LECS is a safe, easy, and beneficial procedure for gastric SMTs. Endoscopy functions to locate the tumor and to support the gastric lumen. The LECS technique, therefore, provides an alternative gastric wedge resection procedure with minimal transformation of the stomach.

## COMMENTS

### Background

Although gastric small mucosal tumors (SMT) have been resected laparoscopically, this type of surgery is associated with two potential problems. Laparoscopy may be unable to determine the location of gastric SMTs, because of their small size or intraluminal growth pattern. In addition, complications may arise during the laparoscopic removal of SMTs located near the cardia or pylorus; these complications can include stenosis or damage to the cardia or pylorus.

### Research frontiers

Laparoscopic-endoscopic cooperative surgery (LECS) is indicated for the removal of SMTs (*e.g.*, leiomyomas, lipomas, and schwannomas), polyps with broad stalks, gastric epithelial tumor degeneration (moderate or severe atypical hyperplasia), lesions with low potential for malignancy (*e.g.*, carcinoid tumors and gastrointestinal stromal tumors), and early-stage, localized gastric carcinomas.

### Innovations and breakthroughs

The authors have developed a technique, called minimally invasive LECS, for removal of SMTs. This paper represents the analysis of findings in 101 patients who successfully underwent LECS for gastric SMT at the Department of General Surgery, Peking Union Medical College Hospital, from June 2006 to April 2013.

### Applications

The study results suggest that the LECS is feasible and safe for patients with gastric SMT. Endoscopic intraoperative localization and support can help preserve the cardia and pylorus during surgery.

### Terminology

LECS represents a technique, called minimally invasive laparoscopic-endoscopic cooperative surgery for removal of gastric small mucosal tumors.

### Peer review

This is a very interesting paper about laparoscopic-endoscopic surgery for gastric submucosal tumor. In this manuscript, the authors analyzed 101 patients who underwent laparoscopic-endoscopic surgery for gastric submucosal tumor. The authors discussed the safety and advantages of minimally invasive laparoscopic-endoscopic cooperative surgery for gastric submucosal tumor. The clinical data was well collected, and the surgical procedures of the patients were well described. The references are updating.

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## Clonal immunoglobulin heavy chain and T-cell receptor $\gamma$ gene rearrangements in primary gastric lymphoma

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### Abstract

**AIM:** To study the diagnostic value of immunoglobulin heavy chain (IgH) and T-cell receptor  $\gamma$  (*TCR- $\gamma$* ) gene monoclonal rearrangements in primary gastric lymphoma (PGL).

**METHODS:** A total of 48 patients with suspected PGL at our hospital were prospectively enrolled in this study from January 2009 to December 2011. The patients were divided into three groups (a PGL group, a gastric linitis plastica group, and a benign gastric ulcer group) based on the pathological results (gastric mucosal specimens obtained by endoscopy or surgery) and follow-up. Endoscopic ultrasonography (EUS) and EUS-guided biopsy were performed in all the patients. The tissue specimens were used for histopathological examination and for *IgH* and *TCR- $\gamma$*  gene rearrangement polymerase chain reaction analyses.

**RESULTS:** EUS and EUS-guided biopsy were successfully performed in all 48 patients. In the PGL group ( $n = 21$ ), monoclonal IgH gene rearrangements were detected in 14 (66.7%) patients. A positive result for each set of primers was found in 12 (57.1%), 8 (38.1%), and 4 (19.0%) cases using FR1/JH, FR2/JH, and FR3/JH primers, respectively. Overall, 12 (75%) patients with mucosal-associated lymphoid tissue lymphoma ( $n = 16$ ) and 2 (40%) patients with diffuse large B-cell lymphoma ( $n = 5$ ) were positive for monoclonal IgH gene rearrangements. No patients in the gastric linitis plastica group ( $n = 17$ ) and only one (10%) patient in the benign gastric ulcer group ( $n = 10$ ) were positive for a monoclonal *IgH* gene rearrangement. No *TCR- $\gamma$*  gene monoclonal rearrangements were detected. The sensitivity of monoclonal IgH gene rearrangements was 66.7% for a PGL diagnosis, and the specificity was 96.4%. In the PGL group, 8 (100%) patients with stage IIE PGL ( $n = 8$ ) and 6 (46.1%) patients with stage IE PGL ( $n = 13$ ) were positive for monoclonal *IgH* gene rearrangements.

**CONCLUSION:** *IgH* gene rearrangements may be associated with PGL staging and may be useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica.

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**Key words:** Immunoglobulin heavy chain; T-cell receptor  $\gamma$ ; Gene rearrangement; Primary gastric lymphoma; Endoscopic biopsy specimen

**Core tip:** In 2003, a new primer system was successfully developed and standardized for the detection of clonally rearranged immunoglobulin (Ig) and T-cell receptor (*TCR*) genes. This study was a prospective analysis of Ig heavy chain (*IgH*) and *TCR- $\gamma$*  gene rearrangements using the new primer system and endoscopic biopsy specimens from patients with suspected primary gastric lymphoma (PGL). Our study revealed that the detec-

tion of monoclonal *IgH* gene rearrangements is useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica. Monoclonal *IgH* gene rearrangements may be associated with PGL staging. The sensitivity and the specificity of *IgH* gene rearrangements for the diagnosis of PGL were 66.7% and 96.4%, respectively.

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## INTRODUCTION

Primary gastric lymphoma (PGL) is a relatively rare tumor type that accounts for 5% of all gastric tumors<sup>[1]</sup>. Gastroscopy and biopsy are the primary methods for diagnosis. However, most PGLs arise in the submucosa, and the diagnosis of PGL by gastroscopy and biopsy is often difficult. An endoscopic presentation of polypoid lesions, flat lesions, enlarged gastric folds, ulcers, erosions, and negative or inconclusive histology may lead clinicians to suspect gastric lymphoma<sup>[2-8]</sup>. It is recommended that biopsy specimens undergo histomorphological, immunohistochemical, and immunophenotypic analyses for a diagnosis of gastric lymphoma. However, these methods may not lead to a diagnosis, especially in the early stages of the disease.

Immunoglobulin (Ig) and T cell receptors (TCRs) are the molecules responsible for B- and T-cell immune responses. The analysis of antigen receptor gene rearrangements by polymerase chain reaction (PCR) is a routine diagnostic tool for lymphoproliferative disorders<sup>[9]</sup>.

Previous studies have demonstrated that Ig gene rearrangements in endoscopic biopsy samples were an additional tool for the diagnosis of gastric mucosal-associated lymphoid tissue (MALT) lymphoma<sup>[10-15]</sup>. In previous studies, the primers and PCR conditions were not standardized. Multiplex PCR assays have been available since 2003, and these assays have been standardized for the detection of clonally rearranged *Ig* and *TCR* genes<sup>[16]</sup>. However, multiplex PCR assays for the detection of Ig heavy chain (*IgH*) and *TCR- $\gamma$*  gene rearrangements in PGL have not been previously reported. The aim of this study was to investigate the detection rate and the diagnostic value of *IgH* and *TCR- $\gamma$*  gene monoclonal rearrangements in PGL endoscopic biopsy specimens.

## MATERIALS AND METHODS

### Patients

A total of 48 patients with suspected PGL at our hospital were prospectively enrolled in this study from January 2009 to December 2011. The patients were divided into three groups based on the pathological results (gastric

mucosal specimens obtained by endoscopy or surgery) and follow-up. The PGL group consisted of 21 patients (14 males, 7 females, a mean age of 51 years, range 20-81 years). The gastric linitis plastica group consisted of 17 patients (11 males, 6 females, a mean age of 53 years, range 17-79 years). The benign gastric ulcer group consisted of 10 patients (7 males, 3 females, a mean age of 47 years, range 22-76 years).

### Methods

Patients who met the criteria for suspected gastric lymphoma (an endoscopic presentation of polypoid lesions, flat lesions, enlarged gastric folds, ulcers, erosions, and negative or inconclusive histology) were included in the study. Patients with palpable superficial lymphadenopathy, obvious mediastinal lymphadenopathy, abnormal total and differential white blood cell counts, and the involvement of other organs in the abdomen were excluded from the study.

Informed consent was obtained from all the patients and this study was approved by the hospital before endoscopic ultrasonography (EUS) and the medical records analysis. EUS and EUS-guided biopsy were performed in all the patients. Overall, 8-10 biopsies were obtained from each patient. The specimens were submitted for histopathological examination. A portion of each specimen was stored at -80 °C for the gene rearrangement analysis by PCR.

DNA was isolated from frozen tissue by cell lysis, phenol extraction, and ethanol precipitation according to standard procedures. Alternatively, reactive DNAzol (Songon Biotech, Shanghai, China) was used according to the manufacturer's specifications. In each experiment, polyclonal DNA (reactive lymphoid tissue) and negative (sterile water) and positive controls were systematically included. To analyze the *IgH* gene, three sets of VH primers and one JH consensus primer were combined in three multiplex tubes. To analyze the *TCR- $\gamma$*  gene, four V $\gamma$  primers and two J $\gamma$  primers were divided into two tubes (Table 1).

The PCR conditions were  $\times$  1 PCR buffer [50 mmol/L KCl, 10 mmol/L Tris (pH, 8.3), 1.5 mmol/L MgCl<sub>2</sub>], 200  $\mu$ mol/L of each deoxynucleotide, 10 pmol of each primer, and 2 U of Taq polymerase (Songon Biotech, Shanghai, China). The total PCR reaction volume was 50  $\mu$ L. The thermal cycling conditions were pre-activation for 7 min at 95 °C, followed by annealing at 60 °C. Each reaction consisted of 35 cycles. The cycles were preceded by an initial denaturation step for 45 s, followed by a terminal extension for 10 min.

Patients with PGL were staged at baseline using computed tomography scans of the neck, the thorax, and the abdomen, followed by EUS and bone marrow biopsy. EUS staging was performed according to the Ann Arbor staging system<sup>[10]</sup>.

## RESULTS

EUS and EUS-guided biopsy were successfully performed in all 48 patients. In the PGL group, monoclonal

Table 1 Primer sequences

Gene	Sequence
IgH tube A	
VH1-FR1	5'GGCCTCAGTGAAGGTCTCCTGCAAG3'
VH2-FR1	5'GTCTGGTCTACGCTGGTGAACCC3'
VH3-FR1	5'CTGGGGGGTCCCTGAGACTCTCTG3'
VH4-FR1	5'CTTCGGAGACCCTGCCCTCACCTG3'
VH5-FR1	5'CGGGGAGTCTCTGAAGATCTCTGT3'
VH6-FR1	5'TCGCAGACCCTCTCACTCACCTGTG3'
JH consensus	5'CTTACCTGAGGAGACGGTGACC3'
IgH tube B	
VH1-FR2	5'CTGGG TCGCA CAGGC CCCTG GACAA3'
VH2-FR2	5'TGGAT CCGTC AGCCC CCAGG GAAGG3'
VH3-FR2	5'GGTCC GCCAG GCTCC AGGGA A3'
VH4-FR2	5'TGGAT CCGCC AGCCC CCAGG GAAGG3'
VH5-FR2	5'GGGTG CGCCA GATGC CCGGG AAAGG3'
VH6-FR2	5'TGGAT CAGGC AGTCC CCATC GAGAG3'
VH7-FR2	5'TTGGG TCGCA CAGGC CCCTG GACAA3'
JH consensus	5'CTTACCTGAGGAGACGGTGACC3'
IgH tube C	
VH1-FR3	5'TGGAG CTGAG CAGCC TGAGA TCTGA3'
VH2-FR3	5'CAATG ACCAA CATGG ACCCT GTGGA3'
VH3-FR3	5'TCTGC AAATG AACAG CCTGA GAGCC3'
VH4-FR3	5'GAGCT CTGTG ACCGC CGCGG ACACG3'
VH5-FR3	5'CAGCA CCGCC TACCT GCAGT GGAGC3'
VH6-FR3	5'GTTCT CCCTG CAGCT GAACT GTGTG3'
VH7-FR3	5'CAGCA CCGCA TATCT GCAGA TCAG3'
JH consensus	5'CTTACCTGAGGAGACGGTGACC3'
TCR- $\gamma$ tube A	
V $\gamma$ 1f	5'GGAAG GCCCC ACAGC RTCIT3'
v $\gamma$ 10	5'AGCATGGGTAAGACAAGCAA3'
J $\gamma$ 1.1/2.1	5'TTACCAGGCGAAGTTACTATGAGC3'
J $\gamma$ :1.3/2.3	5'GTGTGTTCCACTGCCAAAGAG3'
TCR- $\gamma$ tube B	
V $\gamma$ 9	5'CGGCA CTGTC AGAAA GGAATC3'
V $\gamma$ 11	5'CTTC ACITC CACTI TGAAA3'
J $\gamma$ 1.1/2.1	5'TTACCAGGCGAAGTTACTATGAGC3'
J $\gamma$ :1.3/2.3	5'GTGTGTTCCACTGCCAAAGAG3'

The detection of immunoglobulin heavy chain (*IgH*) gene rearrangement using three sets of VH primers and one JH consensus primer combined in three multiplex tubes. The detection of T-cell receptor  $\gamma$  (*TCR- $\gamma$* ) gene rearrangement using four V $\gamma$  primers and two J $\gamma$  primers, which were divided into two tubes.

*IgH* gene rearrangements were detected in 14 (66.7%) patients. Positive results for each set of primers were obtained in 12 (57.1%), 8 (38.1%), and 4 (19.0%) cases using FR1/JH, FR2/JH, and FR3/JH primers, respectively (Figure 1). No patients in the gastric linitis plastica group were positive for a monoclonal *IgH* gene rearrangement. In the benign gastric ulcer group, a monoclonal *IgH* gene rearrangement was detected in one (10%) patient. Overall, no *TCR- $\gamma$*  gene monoclonal rearrangements were detected. For the diagnosis of primary gastric lymphomas, the sensitivity of the monoclonal *IgH* gene rearrangements was 66.7% and the specificity was 96.4%.

In the PGL group, the clinical stage distribution was as follows: IE in 13 patients and IIE in 8 patients. All 8 (100%) patients with stage IIE PGL were positive for monoclonal *IgH* gene rearrangements. Additionally, 6 (46.1%) patients with stage IE PGL were positive for monoclonal *IgH* gene rearrangements. The PGL group consisted of 16 patients with MALT lymphoma and 5

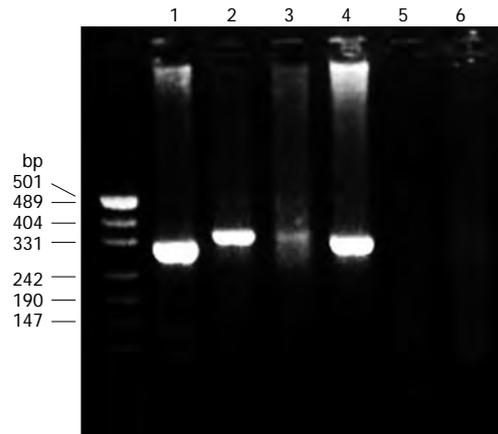


Figure 1 The results of immunoglobulin heavy chain gene rearrangement using FR1/JH primers. Lanes 1, 5, and 6 indicate a positive B-cell gastric lymphoma cell line, a negative control (sterile water), and a polyclonal control, respectively. Lanes 2, 3, and 4 show the presence of a monoclonal rearranged band that is within the expected range of size (242-331 bp).

patients with diffuse large B-cell lymphoma (DLBCL). In the patients with MALT lymphomas, 12 (75%) were positive for monoclonal *IgH* gene rearrangements. In the patients with DLBCL, 2 (40%) were positive for monoclonal *IgH* gene rearrangements.

In the benign gastric ulcer group, patients were treated with a proton-pump inhibitor (esomeprazole 20 mg daily) for 8-16 wk. Repeated endoscopies revealed that the gastric ulcers were completely healed in all the patients. All the patients were followed up for 12-18 mo, and no malignant gastric lesions were found.

## DISCUSSION

Antigen receptor gene rearrangement in lymphocytes is a physiological process. Tumor cells that originate from lymphocytes often carry the same *Ig* and *TCR* gene rearrangements (monoclonal), whereas T and B cells have a unique type of rearrangement in benign lymphoid disorders (polyclonal). Antigen receptor gene rearrangement analysis is useful in differentiating between malignant lymphoproliferative disorders and non-neoplastic lymphoid disorders. There are several PCR targets for the detection of *Ig* and *TCR* rearrangements. Three multiplex PCR assays are available for the detection of clonal *IgH* (VH-JH) rearrangements, and these assays can reliably identify clonal B-cell proliferation and *TCR- $\gamma$*  gene monoclonal rearrangements that occur in most T-cell lymphoid neoplasms<sup>[10]</sup>. *IgH* (VH-JH) and *TCR- $\gamma$*  are the most common PCR targets for detecting *Ig* and *TCR* rearrangements.

In the PGL group, the positive rate of monoclonal *IgH* gene rearrangement in patients with stage IIE PGL was 100% but only 46.1% in patients with stage IE PGL. Previous studies have demonstrated that the positive rate of monoclonal *IgH* gene rearrangements was associated with histological grading (the histological grading of lymphoid infiltrates in the stomach according to the Wother-

spoon-Isaacson histological scoring system). Aiello *et al*<sup>[10]</sup> reported that monoclonal *IgH* gene rearrangements were detected in 64.2%, 41.6%, and 3.1% of samples with histological grading scores of 5, 4, and 0-3, respectively. Additionally, the results of this study suggest that the positive rate of monoclonal *IgH* gene rearrangements may be associated with PGL staging.

In the PGL group, 75% (12/16) of MALT lymphoma patients were positive for monoclonal *IgH* gene rearrangements. However, only 40% (2/5) of DLBCL patients were positive for monoclonal *IgH* gene rearrangements. A previous study reported that the positive rate of monoclonal *IgH* gene rearrangements in MALT lymphoma patients ranged from 62.5%-98.5%<sup>[10-13]</sup>. In the series in this study, a similar rate was observed for monoclonal *IgH* gene rearrangements in MALT lymphoma patients. Thériault *et al*<sup>[17]</sup> reported that the positive rate of monoclonal *IgH* gene rearrangements in DLBCL patients was 78.9% (30/38). However, the specimens in the study included lymph nodes, tonsils, spleens, bone marrow, skin biopsies, and gastrointestinal tract samples. In this study, the positive rate for DLBCL patients was lower than that in the previous study. In addition, recent studies have demonstrated that the detection rate of monoclonal *IgH* gene rearrangement was closely associated with the cell origin of lymphomas<sup>[9]</sup>.

The differential diagnosis between gastric linitis plastica and PGL is not easy for a physician to determine. PGL and gastric linitis plastica usually result in low rates of positive endoscopic biopsies<sup>[18]</sup>. In this series, the first endoscopic biopsies from PGL and gastric linitis plastica patients were all negative. The distinction between PGL and gastric linitis plastica is important because of the different prognoses of these diseases. In this study, 14 (66.7%) patients in the PGL group were positive for a monoclonal *IgH* gene rearrangement; however, no *IgH* gene rearrangements were detected in the patients in the gastric linitis plastica group. One case of gastric cancer was positive for a monoclonal *IgH* gene rearrangement. The patient was diagnosed with carcinoma accompanied by lymphoma<sup>[12]</sup>. This result suggests that the detection of *IgH* gene rearrangements may be helpful in differentiating between PGL and gastric linitis plastica.

According to previous studies, monoclonal rearrangements involving *IgH* genes were detected in 3% of lymphoid disorders that were benign based on clinical and immunohistological evaluations, which is consistent with the findings of this study<sup>[19]</sup>. The majority of these patients suffered from autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and Sjögren's syndrome. These diseases are characterized by polyclonal B-cell activation and autoantibodies<sup>[20]</sup>. In the series in this study, a monoclonal *IgH* gene rearrangement was detected in one patient who was suffering from a benign gastric ulcer. Additionally, this patient had suffered from Sjögren's syndrome for several years.

Gene rearrangement studies can be informative; however, false-positive and false-negative PCR results are problematic. According to previous studies, there are two

main problems with PCR techniques: improper primer annealing and difficulties in discriminating between monoclonal and polyclonal *Ig/TCR* gene rearrangements<sup>[16,21,22]</sup>. Single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis, heteroduplex analysis, or gene scanning may be performed to reduce false-positive and false-negative rates<sup>[16,23-26]</sup>.

In this study, there was one patient with false-negative results in the PGL group. The three endoscopic biopsies for this patient were negative, but the PCR results for a monoclonal *IgH* gene rearrangement were always positive. Autoimmune diseases were excluded. The patient was followed up for 7 mo and was diagnosed with PGL based on the fourth endoscopic biopsy. In addition, Fend *et al*<sup>[14]</sup> reported that the detection of clonal rearrangements in the biopsy specimens from two patients preceded the histological diagnosis of lymphoma by several mo. Because the detection of clonal rearrangements can precede a histological diagnosis, we suggest that patients with suspected PGL and positive results for *IgH* rearrangements need close follow-up.

In conclusion, the presence of an *IgH* gene rearrangement is useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica. Additionally, *IgH* gene rearrangements may be associated with PGL staging.

## COMMENTS

### Background

Primary gastric lymphoma (PGL) is a relatively rare tumor type. The diagnosis of PGL by gastroscopy and biopsy is often difficult. The analysis of antigen receptor gene rearrangements by polymerase chain reaction is a routine diagnostic tool for lymphoproliferative disorders. Previous studies have demonstrated that the positive rate of gene rearrangement was relatively high in PGL patients; however, no prospective studies of gene rearrangement in PGL patients have been reported.

### Research frontiers

Studies are being performed to assess the diagnostic value of immunoglobulin heavy chain (*IgH*) and T-cell receptor  $\gamma$  (*TCR-\gamma*) gene monoclonal rearrangements in PGL patients.

### Innovations and breakthroughs

This study was a prospective analysis of *IgH* and *TCR-\gamma* gene rearrangements in endoscopic biopsy specimens from patients with suspected PGL. According to the pathological results, 48 patients with suspected PGL were divided into three groups, including a PGL group, a gastric linitis plastica group, and a benign gastric ulcer group. The study revealed that the detection of monoclonal *IgH* gene rearrangements is useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica. Additionally, these gene rearrangements may be associated with PGL staging.

### Applications

The results of this study may encourage the detection of *IgH* gene rearrangements for a diagnosis of PGL and for differentiating between PGL and gastric linitis plastica.

### Terminology

Antigen receptor gene rearrangement is a physiological process. Tumor cells that originate from lymphocytes often carry the same *IgH* and *TCR* gene rearrangements (monoclonal), whereas T and B cells have a unique type of rearrangement in benign lymphoid disorders (polyclonal). Antigen receptor gene rearrangement analysis is useful in differentiating between malignant lymphoproliferative disorders and non-neoplastic lymphoid disorders.

### Peer review

This paper includes interesting results and presents an acceptable case for publication because few reports have been published on this subject in China.

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## Application value of multi-slice spiral computed tomography for imaging determination of metastatic lymph nodes of gastric cancer

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Then, the findings were compared with the postoperative pathological results.

**RESULTS:** Among 605 lymph nodes, 358 were confirmed as metastatic, accounting for 59.2%. A total of 535 lymph nodes were detected in original axis images combined with multiplanar reconstruction images of MSCT. The metastatic lymph nodes had specific signs in computed tomography. This study showed that the long diameter of lymph nodes  $\geq 8$  mm indicated metastasis; the sensitivity and specificity were 79.6% and 78.8%, respectively. The difference of the mean value of lymph node enhancement density  $\geq 80$  Hu indicated metastasis; the sensitivity and specificity were 81.6% and 75.6%, respectively. The ratio of short diameter to long diameter of lymph nodes  $\geq 0.7$  indicated metastasis; the sensitivity and specificity were 85.6% and 71.8%, respectively.

**CONCLUSION:** MSCT is a non-invasive and reliable method for preoperative examination of gastric cancer. Sensitivity and specificity for prediction of lymph node metastasis are high.

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**Key words:** X-ray computer; Gastric cancer; Metastatic lymph nodes

### Abstract

**AIM:** To evaluate the application value of multi-slice spiral computed tomography (MSCT) for imaging determination of metastatic lymph nodes of gastric cancer and to explore reasonable diagnostic criteria.

**METHODS:** Sixty patients with gastric cancer underwent 64 MSCT scans before operation. Gastric cancer samples and perigastric lymph nodes were obtained after operation, formalin fixation and haematoxylin-eosin staining. The metastatic conditions of gastric cancer and perigastric lymph nodes were determined under a light microscope. A total of 605 lymph nodes were grouped and assessed according to distribution, size, shape and degree of lymph node enhancement.

**Core tip:** Gastric cancer is one of the most common malignant tumours of the digestive system. In recent years, individualised surgical therapy has been applied for gastric cancer. This study plan explored the distribution, size, shape and enhancement characteristics of metastatic lymph nodes. It also provided a basis for determining lymph node metastasis before surgery by retrospectively analyzing multi-slice spiral computed tomography manifestations of lymph nodes of patients with gastric cancer after surgery in the hospital. The

findings were compared with the pathological results.

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## INTRODUCTION

Gastric cancer is one of the most common malignant tumours of the digestive system. In recent years, individualised surgical therapy has been applied for gastric cancer. The choice of reasonable surgical methods for different stages of gastric cancer depends on accurate preoperative diagnosis. The traditional diagnostic methods of gastric cancer include gastrofiberscope and barium meal of the upper gastrointestinal tract. However, these two methods have certain limitations in the diagnosis of gastric cancer. They cannot clearly display the gastric wall structure, and their ability to determine the presence or absence of adjacent organ invasion, distant metastasis and lymph node metastasis is limited. Multi-slice spiral computed tomography (MSCT) has been used to conduct TM preoperative staging among many patients with gastric cancer in recent years. Original axial images combined with multiplanar reconstruction (MPR) reorganised images are used to carry out preoperative assessment. The assessment includes the location, extent, depth of invasion of gastric cancer, relationship with adjacent organs and metastasis of abdominal organs. Satisfactory results have been achieved. Research on lymph node metastasis of gastric cancer before surgery has shown that a unified standard for determining lymph node metastasis by MSCT is not available worldwide. Lymph node metastasis is a major metastatic mechanism of gastric cancer. Seto *et al*<sup>[1]</sup> reported that the rate of lymph node metastasis of early gastric cancer is 5.7%-29.0%, and the rates are 0.0%-6.4% and 9.7%-24.3% for early gastric intramucosal carcinoma and gastric submucosal carcinoma, respectively. Yasuda *et al*<sup>[2]</sup> showed that the rate of lymph node metastasis of early gastric cancer is 8.9%, and the rates are 2.5% and 17.6% for early gastric intramucosal carcinoma and gastric submucosal carcinoma, respectively. Okusa *et al*<sup>[3]</sup> proposed the concept of metastatic lymph node ratio (MLR). The results of numerous studies worldwide<sup>[4,5]</sup> have shown that MLR is one of the independent prognostic factors of survival of patients with gastric cancer, which is closely correlated with the five-year survival rate of patients with gastric cancer. Both the Union for International Cancer Control (UICC) and the Japanese General Rules for Gastric Cancer Study consider that lymph node metastasis is an independent and important factor for predicting the prognosis of patients with gastric cancer<sup>[6,7]</sup>. To date, radical gastrectomy of

gastric cancer with radical lymph node excision adjacent to the stomach has achieved better therapeutic effects in the surgical therapy of gastric cancer<sup>[8]</sup>. The presence or absence of lymph node metastasis, as well as the degree and extent of metastasis, is directly correlated with the choice of therapeutic methods and prognostic evaluation of patients with gastric cancer, which are primary indices for surgical approach selection. Radical excision of metastatic lymph nodes has important clinical significance and prognostic value in patients with gastric cancer. Radical excision of metastatic lymph nodes may affect the immune function in patients and lead to increased surgical trauma, which cannot improve curative effect. Therefore, confirming the presence or absence of lymph node metastasis of gastric cancer before surgery is important for preoperative staging, formulation of clinical therapeutic schedule and prognosis evaluation<sup>[9]</sup>. MSCT has the advantages of rapid scanning speed, high resolution ratio and convenient image reconstruction, which can estimate lymph nodes by thin-layer scanning and reconstruction technique and direct clinical staging<sup>[10-12]</sup>. The standard for determining metastasis of lymph nodes by enhancement characteristics and lymph node size displayed by MSCT is the focus of studies and controversies among many researchers. Too high or too low MSCT staging may appear during clinical application because of different standards of size and morphology of lymph nodes and different sizes of lymph nodes in different positions<sup>[13]</sup>. Thus, this study plan explored the distribution, size, shape and enhancement characteristics of metastatic lymph nodes. It also provided a basis for determining lymph node metastasis before surgery by retrospectively analyzing MSCT manifestations of lymph nodes of patients with gastric cancer after surgery in the hospital. The findings were compared with the pathological results.

## MATERIALS AND METHODS

### Objectives

Sixty patients with gastric cancer who were hospitalised in the First Affiliated Hospital of Jilin University and underwent MSCT scanning before operation from February 2010 to October 2011 were included the study. The patients comprised 48 male and 12 female with a mean age of 59.5 years (36-78 years). A total of 51 patients were confirmed to have metastatic lymph nodes of gastric cancer after operation, whereas no metastatic lymph nodes were observed in nine patients. The histopathological types included poorly differentiated adenocarcinoma (39 patients), moderately differentiated tubular adenocarcinoma (17 patients) and signet-ring cell carcinoma (4 patients).

### Methods

All the patients were asked to fast for 6-8 h before scanning. They were treated with intramuscular injection of 654-2 (20 mg) and oral administration of warm water 10 min before scanning. The patients were scanned in

supine position using 64-MSCT (Siemens, Germany). Scanning parameters were as follows: spiral collimation,  $64 \times 0.625$ ; thickness of every layer, 5 mm; interval thickness of every layer, 5 mm; speed of bed movement, 12 mm/s; tube voltage, 120 kV and tube current, 260-320 mAs. During plain, arterial and venous scanning phases, the extent of scanning was from the lower oesophagus to the level of inferior pole of kidney, including the whole gastric area. During equilibrium phase, the extent of scanning was from the diaphragmatic dome to the pelvic cavity. Each scan was performed during a breath hold at the end of inspiration. Anconal venous transfusion of non-ionic contrast medium (OmniPaque 300 or Ultravist 300; 80-100 mL) with high pressure injector was used during the enhanced scanning. The rate of injection was 3.0 mL/s, and the starting times of arterial scanning, venous scanning and equilibrium phases were 25, 35 and 60 s after the beginning of injection. After scanning, the original data were treated with thin-slice reconstruction (1 mm; the interval between two adjacent slices was 1 mm). The images of all patients underwent MPR.

### Image analysis

Two doctors with years of experience on abdominal image diagnosis analysed and treated the images. According to anatomic sites, original axis images combined with MPR were applied to observe various indices of lymph nodes, including distribution, number, size, shape and degree of lymph node enhancement.

### Evaluation of results

Evaluation criteria of CT signs: The lymph nodes were divided into three groups according to the long diameter:  $\geq 5$  mm group,  $\geq 8$  mm group and  $\geq 10$  mm group. Ten points of non-cystic area in each detected lymph node were randomly selected in MSCT. The mean difference of CT value during venous scanning phase and plain scanning was measured. Then, the lymph nodes were divided into three groups according to the degree of enhancement: the difference of mean value of enhancement density  $\geq 100$  Hu group,  $\geq 80$  Hu group and  $\geq 40$  Hu group. The lymph nodes were divided into two groups according to the ratio of short diameter to long diameter:  $\geq 0.5$  group and  $\geq 0.7$  group.

### Pathological criteria

Gastric cancer samples and perigastric lymph nodes were obtained after operation, formalin fixation and haematoxylin-eosin staining. The metastatic conditions of gastric cancer and perigastric lymph nodes were determined under a light microscope.

### Statistical analysis

All the data were analysed using SPSS17.0 software. *K* test was used to evaluate the consistency of metastatic lymph nodes of gastric cancer between MSCT and postoperative pathological diagnosis. Kappa coefficient within 0.71-1.00, 0.41-0.70 and  $\leq 0.4$  indicated strong, general

and weak consistencies, respectively.

## RESULTS

Comparison of different long diameters of lymph nodes displayed by MSCT and postoperative pathological results for determination of lymph node metastasis.

A total of 605 lymph nodes were cleaned up in the operation, among which 358 were diagnosed as metastatic by postoperative pathological examination, accounting for 59.2%. Original axis images combined with MPR images found 535 lymph nodes. The lymph nodes could be analysed and determined with a group of lymph nodes as a unit in MSCT images because the lymph nodes were excised with a group as a unit instead of surgically operated, according to the images. A single lymph node could not be specifically studied. Thus, the ability of MSCT to determine the specificity and sensitivity of lymph nodes could be improved as a whole in the study. The data from the different groups were compared with the pathological results. The consistency of lymph node diameter  $\geq 5$  mm and the postoperative pathological results was general ( $K = 0.464$ ). The consistency of lymph node diameter  $\geq 8$  mm and the postoperative pathological results was strong ( $K = 0.831$ ). The consistency of lymph node diameter  $\geq 10$  mm and the postoperative pathological results was weak ( $K = 0.232$ ) (Table 1).

Comparison of different degrees of lymph node enhancement displayed by MSCT and postoperative pathological results for determination of lymph node metastasis.

The consistency of the difference of the mean value of enhancement density  $\geq 80$  Hu and the postoperative pathological results was strong ( $K = 0.849$ ). The consistency of the difference of the mean value of enhancement density  $\geq 100$  Hu and the postoperative pathological results was weak. The consistency of the difference of the mean value of enhancement density  $\geq 40$  Hu and the postoperative pathological results was weak ( $K < 0.40$ ) (Table 1).

Comparison of different ratios of short diameter to long diameter of lymph nodes displayed by MSCT and postoperative pathological results for determination of lymph node metastasis.

The consistency of the ratio of short diameter to long diameter of lymph nodes  $\geq 0.7$  and the postoperative pathological results was strong ( $K = 0.873$ ). The consistency of the ratio of short diameter to long diameter of lymph nodes  $> 0.5$  and the postoperative pathological results was general ( $K = 0.513$ ) (Table 1).

The diagnostic criteria of metastatic lymph nodes in MSCT for patients with gastric cancer included long diameter of lymph nodes  $\geq 8$  mm, the ratio of short diameter to long diameter of lymph nodes  $\geq 0.7$  and the difference of the mean value of enhancement density  $\geq 80$  Hu. Compared with the postoperative pathological results, the sensitivities and specificities of MSCT for detection of lymph nodes adjacent to the celiac artery and lesser curvature were 89.4% and 90.3%, respectively. The

**Table 1 Comparison of different long diameters of lymph nodes displayed, degrees of lymph node enhancement displayed and ratios of short diameter to long diameter of lymph nodes displayed by multi-slice spiral computed tomography and postoperative pathological results for determination of lymph node metastasis**

		Number of lymph nodes detected by MSCT	K value	Sensitivity	Specificity
Diameter of lymph nodes	≥ 5 mm	470	0.464	88.5%	60.1%
	≥ 8 mm	317	0.831	79.6%	78.8%
	≥ 10 mm	128	0.232	48.6%	93.5%
Difference of enhancement of lymph nodes	≥ 40 Hu	495	0.397	89.3%	65.5%
	≥ 80 Hu	379	0.849	81.6%	75.6%
	≥ 100 Hu	197	0.335	53.8%	95.5%
Ratio of short diameter to long diameter of lymph nodes	≥ 0.5	447	0.513	94.3%	57.3%
	≥ 0.7	375	0.873	85.6%	71.8%

MSCT: Multi-slice spiral computed tomography.

**Table 2 Comparison of sensitivities and specificities of metastatic lymph nodes in different groups displayed by multi-slice spiral computed tomography**

Positions of lymph nodes	Specificity	Sensitivity
Right area of cardiac orifice	79.2%	57.8%
Left area of cardiac orifice	81.3%	73.5%
Lesser curvature	79.6%	90.3%
Greater curvature	87.5%	45.0%
Superior area of pylorus	88.1%	76.7%
Inferior area of pylorus	78.0%	81.9%
Adjacent to left gastric artery, common hepatic artery, and arteria coeliaca	83.2%	89.4%

detection rates of lymph nodes in the right area of the cardiac orifice and adjacent to the greater curvature were 57.8% and 45.0%, respectively (Table 2).

## DISCUSSION

Tumour-node-metastasis staging system is one of the most commonly used staging systems, and is accepted and maintained by the UICC and the American Joint Committee on Cancer<sup>[14]</sup>. Lymph node metastasis is a major metastatic pathway of gastric cancer. The metastatic rates of lymph nodes in gastric cancer at early and progressive stages are 10% and 74.8%, respectively<sup>[15]</sup>. The lymph node size determines the lymph node metastasis. Lymph node diameter > 10 mm is used as one of the criteria to diagnose lymph node metastasis of gastric cancer by MSCT. Some researchers have suggested that the lymph node diameter > 5 mm can be used as a criterion of lymph node metastasis of gastric cancer<sup>[16,17]</sup>. Dux proposed that all the lymph nodes detected by MSCT could be considered as lymph node metastasis. Some researchers have considered that the short diameter of perigastric lymph nodes > 6 mm or the short diameter of lymph nodes adjacent to the stomach > 8 mm should be regarded as metastasis<sup>[18]</sup>. Moreover, other researchers believe that determining lymph node metastasis by imaging is not sufficient because the sensitivity of revealing small lymph nodes by imaging is low. This study selected 5, 8 and 10 mm (long diameter of lymph nodes) as threshold values of lymph node metastasis, and then a comparative

study was performed. The long lymph node diameter of 8 mm was determined as the threshold value of lymph node metastasis after statistical analysis. Compared with the postoperative pathological results, the sensitivity and specificity were more reasonable.

The degree of lymph node enhancement is an important index to determine lymph node metastasis. The perigastric lymph nodes have a specific blood supply, and this blood supply is abundant when metastasis of lymph nodes occurs. After MSCT enhancement, obvious lymph node enhancement is displayed, but the non-metastatic lymph nodes exhibit absence of enhancement or mild enhancement. Fukuya *et al.*<sup>[9]</sup> posited that high density or peripheral high density and central low density of metastatic lymph nodes of gastric cancer show moderate or obvious enhancement, but non-metastatic lymph nodes show no enhancement or mild enhancement. Some researchers believe that CT value ≥ 25 Hu during plain scanning phase, CT value ≥ 70 Hu during arterial scanning phase or CT value ≥ 80 Hu during venous scanning phase is a criterion of positive lymph nodes, which can significantly improve the diagnostic rate of lymph node metastasis<sup>[20,21]</sup>. In this study, the consistency of the difference of the mean value of enhancement density ≥ 80 Hu and the postoperative pathological results was strong ( $K = 0.849$ ). The sensitivity and specificity were 81.6% and 75.6%, respectively. Selecting 80 Hu is recommended to determine the threshold value of metastatic lymph nodes of gastric cancer.

The morphology of lymph nodes displayed by MSCT is also an important index to determine lymph node metastasis. Certain exogenous and expansible growth characteristics are shown in lymph nodes when lymph node metastasis occurs. These characteristics contribute to the round or oval morphology of lymph nodes, and the expansible growth of lymph nodes is not balanced so the margin of metastatic lymph nodes is irregular and appears blurred. Thus, the morphology of lymph nodes is one of the references in determining lymph node metastasis by imaging. Fukuya *et al.*<sup>[9]</sup> posited that the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer was  $(0.81 \pm 0.15)$ , but the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer was  $(0.57 \pm 0.15)$ . This study showed

that the consistency of the ratio of short diameter to long diameter of lymph nodes  $\geq 0.7$  and the postoperative pathological results was strong ( $K = 0.873$ ). When the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer  $\geq 0.7$  was selected as a criterion, the sensitivity and specificity were 85.6% and 71.8%, respectively. This result showed that the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer  $\geq 0.7$  in MSCT is more reasonable as a criterion.

The detection rate of lymph nodes in MSCT is correlated with the location of lymph nodes. Some studies have reported that the detection rates of MSCT for mesenteric lymph nodes and lymph nodes adjacent to the aorta were the highest, followed by the lymph nodes adjacent to the lesser curvature and celiac artery. The detection rates of MSCT for lymph nodes adjacent to the ligamentum hepatoduodenale and common hepatic artery were relatively low, and the detection rate of MSCT for lymph nodes adjacent to the nidi was low<sup>[6]</sup>. The statistical results of the study showed that the sensitivities of MSCT for lymph nodes adjacent to the celiac artery and lesser curvature were 89.4% and 90.3%, respectively. However, the detection rates of MSCT for lymph nodes adjacent to the right area of cardiac orifice and greater curvature were relatively low, especially the lymph nodes adjacent to the greater curvature (45.0%). These results were attributed to the lymph nodes adjacent to the lesser curvature, which were near the stomach wall. The lymph nodes showed soft tissue density; after filling the stomach with water, the lymph nodes were easily foiled. The lymph nodes adjacent to the celiac artery were near the vessels, and lymph node enhancement and vascular enhancement were not significantly co-gradient. They were easily distinguished according to density; thus, the sensitivity of lymph nodes adjacent to the celiac artery was high. The perigastric fat content is one of the important factors affecting the detection rate of lymph nodes by MSCT. In this study, the stomachs of the patients were filled after drinking water, thereby reducing the fat adjacent to the greater curvature and surrounding organs. This phenomenon caused the surrounding lymph nodes not to be displayed, which was one of the main reasons for the low detection rate of lymph nodes adjacent to the greater curvature. In addition, the resolution ratio of MSCT for lymph nodes adjacent to the pancreas was relatively low because most of the patients with gastric cancer were elderly. Their pancreas atrophied, showing nodositas. The densities of lymph nodes adjacent to the pancreas during plain scanning and enhancement scanning were similar to the density of pancreatic substance. The lymph nodes were easily diagnosed as swelling lymph nodes, so determining swelling lymph nodes adjacent to the pancreas by MSCT has a certain limitation.

In conclusion, conducting MSCT examination before operation among patients with gastric cancer, as well as observing and measuring the size, degree of enhancement and morphology of lymph nodes, is helpful in

determining lymph node metastasis before operation. The results will provide references for staging of gastric cancer before operation and for choosing a therapeutic schedule.

## COMMENTS

### Background

Lymph node metastasis is the most important metastatic methods of gastric cancer; it is also one of the important factors affecting the prognosis of patients. The presence or absence of lymph node metastasis, as well as the degree and extent of metastasis, is directly correlated with the choice of therapeutic methods and prognostic evaluation of gastric cancer. Considerable studies exist on predicting lymph node metastasis of gastric cancer before surgery by multi-slice spiral computed tomography (MSCT). A number of predictive criteria are available, including distribution, size and enhancement of lymph nodes, but they are not unified and acknowledged diagnostic criteria.

### Research frontiers

MSCT is widely used for diagnosis of preoperative staging of gastric cancer, especially 64-row MSCT or above. This method significantly improves the resolution ratio of images, achieves complete accordance of resolution ratios on axial view, coronal view, sagittal view, inclined plane and curved surface, and provides valuable information for displaying metastatic lymph nodes.

### Innovations and breakthroughs

This study investigated the enhancement characteristics of metastatic lymph nodes displayed by MSCT and the relationship between the size of lymph nodes and metastasis. This research also determined the effects of different positions of lymph nodes on the detection rate of MSCT and the relationship between morphology of lymph nodes and metastasis. Preoperative MSCT scanning is one of the important tools used to evaluate the prognosis of patients with gastric cancer.

### Applications

N-stage and prognostic evaluation of gastric cancer were performed and applied in the First Affiliated Hospital of Jilin University according to the diagnostic criteria of lymph node metastasis of gastric cancer before surgery designed in this study. The accurate rate of preoperative MSCT for evaluation of lymph node metastasis of gastric cancer was high based on the pathological results of more than 60 patients with gastric cancer after surgery.

### Peer review

Application value of multi-slice spiral computed tomography for imaging determination of metastatic lymph nodes of gastric cancer. In this article, authors try to evaluate the application value of multi-slice spiral computed tomography for imaging determination of metastatic lymph nodes of gastric cancer and to explore reasonable diagnostic criteria. As is known, MSCT has been used to conduct TM preoperative staging among many patients with gastric cancer in recent years.

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## Induction of clinical response and remission of inflammatory bowel disease by use of herbal medicines: A meta-analysis

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### Abstract

**AIM:** To evaluate the efficacy and tolerability of herbal medicines in inflammatory bowel disease (IBD) by conducting a meta-analysis.

**METHODS:** Electronic databases were searched for studies investigating efficacy and/or tolerability of herbal medicines in the management of different types of IBD. The search terms were: "herb" or "plant" or "herbal" and "inflammatory bowel disease". Data were collected from 1966 to 2013 (up to Feb). The "clinical response", "clinical remission", "endoscopic response", "endoscopic remission", "histological response", "histological remission", "relapse", "any adverse events", and "serious

adverse events" were the key outcomes of interest. We used the Mantel-Haenszel, Rothman-Boice method for fixed effects and DerSimonian-Laird method for random-effects. For subgroup analyses, we separated the studies by type of IBD and type of herbal medicine to determine confounding factors and reliability.

**RESULTS:** Seven placebo controlled clinical trials met our criteria and were included (474 patients). Comparison of herbal medicine with placebo yielded a significant RR of 2.07 (95%CI: 1.41-3.03,  $P = 0.0002$ ) for clinical remission; a significant RR of 2.59 (95%CI: 1.24-5.42,  $P = 0.01$ ) for clinical response; a non-significant RR of 1.33 (95%CI: 0.93-1.9,  $P = 0.12$ ) for endoscopic remission; a non-significant RR of 1.69 (95%CI: 0.69-5.04) for endoscopic response; a non-significant RR of 0.64 (95%CI: 0.25-1.81) for histological remission; a non-significant RR of 0.86 (95%CI: 0.55-1.55) for histological response; a non-significant RR of 0.95 (95%CI: 0.52-1.73) for relapse; a non-significant RR of 0.89 (95%CI: 0.75-1.06,  $P = 0.2$ ) for any adverse events; and a non-significant RR of 0.97 (95%CI: 0.37-2.56,  $P = 0.96$ ) for serious adverse events.

**CONCLUSION:** The results showed that herbal medicines may safely induce clinical response and remission in patients with IBD without significant effects on endoscopic and histological outcomes, but the number of studies is limited to make a strong conclusion.

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**Key words:** Herbal medicine; Inflammatory bowel disease; Efficacy; Relapse; Adverse events; Meta-analysis

**Core tip:** Meta-analysis of seven controlled trials involving 474 patients demonstrated that herbal medicines may safely induce clinical response and remission in patients with inflammatory bowel disease without significant effects on endoscopic and histological outcomes. The results of sub-analyses based on plant

type demonstrated that induction of clinical remission was obtained only by *Artemisia absinthium* and *Boswellia serrata* and induction of clinical response was gained by only *Aloe vera* and *Triticum Aestivum*. *Boswellia serrata* in one study evaluating recurrence rate did not cause prevention of relapse. Induction of adverse events by none of the plants was significant compared to that of placebo.

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of gastrointestinal tract with two major types including ulcerative colitis (UC) and Crohn's disease (CD) and some atypical forms like collagenous colitis and intractable colitis. Many etiological factors have been implicated to play role in IBD; the most important one is immunological disturbances. Different drug categories are used for the management of IBD like aminosalicylates<sup>[1]</sup>, corticosteroids<sup>[2]</sup>, anti-tumor necrosis factor alpha drugs<sup>[3,4]</sup>, antibiotics<sup>[5,6]</sup>, probiotics<sup>[7,8]</sup>, and immunosuppressants<sup>[9]</sup>. Because of lack of desirable efficacy and poor tolerability of these drugs, approach toward complementary and alternative medicines especially herbal medicines for the management of IBD are increasing<sup>[10,11]</sup>. Besides many *in vivo* studies<sup>[12-14]</sup>, the efficacy and tolerability of herbal medicines in IBD have been investigated through several clinical trials. In this paper, all of these clinical trials were retrieved and a meta-analysis was performed to obtain conclusive results about efficacy and tolerability of herbal medicines for the management of IBD.

## MATERIALS AND METHODS

### Methods

The procedures performed in this meta-analysis are in accordance with recent guidelines for the reporting of meta-analysis (PRISMA guidelines).

### Data sources and searches

PubMed, Scopus, Web of Science, and Cochrane Central Register of Controlled Trials were searched for studies evaluating efficacy and/or tolerability of herbal medicines in any types of IBD. Data were collected from 1966 to 2013 (up to Feb). The search terms were: "herb" or "plant" or "herbal" and "inflammatory bowel disease". There was no language restriction. The reference list from retrieved articles was also reviewed for additional

applicable studies.

### Study selection

Controlled trials evaluating the efficacy and/or tolerability of herbal medicines in patients with any types of IBD were considered. The "clinical response", "clinical remission", "endoscopic response", "endoscopic remission", "histological response", "histological remission", "relapse", "any adverse events", and "serious adverse events" were the key outcomes of interest. All published studies as well as abstracts presented at meetings were evaluated. Two reviewers independently examined the title and abstract of each article to eliminate duplicates, reviews, case studies, and uncontrolled trials.

The reviewers independently extracted data on patients' characteristics, therapeutic regimens, dosage, trial duration, and outcome measures. There was no disagreement between reviewers.

### Quality assessment

Jadad score, which indicates the quality of the studies based on their description of randomization, blinding, and dropouts (withdrawals) was used to assess the methodological quality of trials<sup>[15]</sup>. The quality scale ranges from 0 to 5 points with a low quality report of score 2 or less and a high quality report of score at least 3.

### Statistical analysis

Data from selected studies were extracted in the form of 2 × 2 tables by study characteristics. Included studies were weighted and pooled. Data were analyzed using StatsDirect software version 2.7.9. RR and 95%CI were calculated using Mantel-Haenszel, Rothman-Boice (for fixed effects) or Der Simonian-Laird (for random effects) methods. The Cochran *Q* test was used to test heterogeneity and *P* < 0.05 considered significant. In case of heterogeneity or few included studies, the random effects model was used. Funnel plot was used as publication bias indicator.

## RESULTS

The electronic searches yielded 1224 items; 698 from PubMed, 5 from Cochrane Central, 35 from Web of Science, and 355 from Scopus. Of those, 41 trials were scrutinized in full text.

Thirty four reports were considered ineligible. Thus, 7 trials were included in the analysis represented 474 patients (Figure 1)<sup>[16-22]</sup>. From these 7 studies, 5 obtained Jadad score of 4 or more<sup>[16,17,20-22]</sup> and remaining two gained Jadad score of 2<sup>[18,19]</sup> (Table 1). Among studies included, 3 investigated the efficacy and/or tolerability of herbal medicines in CD<sup>[18-20]</sup>, 3 in UC<sup>[16,17,22]</sup> and 1 in collagenous colitis<sup>[21]</sup>. Five plants were investigated in 7 included studies: *Aloe vera*<sup>[16]</sup>, *Andrographis paniculata*<sup>[17]</sup>, *Artemisia absinthium*<sup>[18,19]</sup>, and *Boswellia serrata*<sup>[20,21]</sup>, and *Triticum aestivum*<sup>[22]</sup>. Induction of treatment was investigated in six studies and duration of these studies is between 4

Table 1 Characteristics of studies included in the meta-analysis

Study	Scientific name of plant(s)	Study design	Method of randomization	Blindness	Withdrawal Jadad score	Inclusion criteria	Exclusion criteria	Interventions	Concomitant medications	Duration	Outcomes
Sandborn <i>et al.</i> <sup>[17]</sup>	<i>Andrographis paniculata</i>	Randomized, placebo-controlled, double-blind	Block randomization schedule	Double-blind	4	Patients with at least 18 yr of age and confirmed diagnosis of mildly to moderately active UC (Mayo Score of 4-10 points and endoscopic subscore of at least 1) while receiving either oral mesalamine (or equivalent sulfasalazine, medications balsalazide, and olsalazine) for at least 4 wk or no medical therapy	Patients with CD or indeterminate colitis, severe UC (Mayo Score of 11 or 12 points, toxic mega-colon, toxic colitis), previous colonic surgery or probable requirement for intestinal surgery within 12 wk, enteric infection within 2 wk, a history of tuberculosis, a positive chest X-ray or tuberculin protein-purified derivative skin test, active infection with hepatitis B or any infection with hepatitis C, infection with human immunodeficiency virus, cancer within 5 yr, inadequate bone marrow, hepatic, or renal function, a history of alcohol or drug abuse that would interfere with the study, significant concurrent medical diseases, allergy to plants in the Acanthaceae family, women who were pregnant or breastfeeding, receiving oral or rectal steroids within 1 mo, rectal mesalamine within 1 wk, azathioprine, anti-tumor necrosis factor agents, or immunosuppressive therapy within 6 wk	Group 1: Capsules containing 1200 or 1800 mg <i>Andrographis paniculata</i> ethanol (male/female: 81/68); 1 cap <i>tds</i> Group 2: The same capsules without herbal extract. [ <i>n</i> = 149 (male/female: 41/34)]; 1 cap <i>tds</i>	Mesalazine	8 wk	(1) Clinical response (a decrease from baseline in the total Mayo Score by at least 3 points and at least 30% with an accompanying decrease in rectal bleeding subscore of at least 1 point or an absolute rectal bleeding subscore of 0 or 1 point); (2) Clinical remission (a total Mayo Score of 2 points or lower, with no individual subscore exceeding 1 point); (3) Mucosal healing (a decrease from baseline in the endoscopy subscore by at least 1 point and an absolute endoscopy subscore of 0 or 1 point)
Holtmeier <i>et al.</i> <sup>[20]</sup>	<i>Boswellia serrata</i>	Randomized, placebo-controlled, double-blind	A computer generated randomization scheme: In blocks of four	Double-blind	4	Outpatients between 18 and 75 yr with a history of CD currently in remission with at least two documented relapses during the last 4 yr, one within the last 18 mo, or a recent resection (fibrotic strictures without inflammation were not considered a relapse); CDAI < 150 and no symptoms suspicious of activity for the previous 28 d	CDAI of > 150 at screening and at baseline visit ( $\geq 28$ d apart); severe fistulizing CD; abscesses; symptomatic stenoses; any condition that places the patient at an undue risk; surgical bowel resections within 3 mo, short bowel syndrome; total proctocolectomy; serious infections, nutritionally compromised patients requiring enteral or parenteral therapy; severe hypertension, chronic liver disorder; impaired renal function; myocardial infarction < 3 mo, cerebral blood flow disturbances or cerebral infarction < 6 mo; any history of malignancy within the past 5 yr (except for squamous or basal cell carcinoma of the skin); subjects with severe psychiatric illnesses, inability to give informed consent; and history of severe alcoholism and drug abuse; taken monoclonal antibody therapy (e.g., infliximab) within 12 mo, immunosuppressives (azathioprine/6-mercaptopurine, cyclosporine, methotrexate) within 4 mo, or corticosteroids, mesalamine/sulfasalazine, or <i>Boswellia serrata</i> within 6 wk prior to randomization	Group 1: Capsules containing 400 mg 8% ethanol extract of <i>Boswellia serrata</i> resin. [ <i>n</i> = 42 (male/female: 13/29)]. 2 caps <i>tds</i> Group 2: The same capsules without herbal extract. [ <i>n</i> = 40 (male/female: 15/25)]. 2 caps <i>tds</i>	ND	52 wk	(1) Maintenance of remission (maintenance of CDAI < 150 throughout study); (2) Relapse (relapse was defined as both a CDAI score > 150 points and an increase in the CDAI score of $\geq 70$ points)

Krebs <i>et al</i> <sup>[18]</sup>	<i>Artemisia absinthium</i>	Randomized, open label	ND	Unblinded	Not any	2	Patients between 18 and 80 yr with CDAI $\geq$ 200 at least for 3 mo receiving CD treatments with 5-aminosalicylates stable dose for at least 4 wk, azathioprine stable dose for 8 wk, methotrexate stable dose for 6 wk or steroids with stable dose in the range of 20-30 mg (equivalent to dexamethasone)	Treatment with TNF- $\alpha$ inhibitors such as infliximab; Patients with serious pathological findings in ECG, liver, kidney and heart functions, or coexisting organic diseases such as a history of cancer, asthma or other autoimmune disease, or pregnancy; opinion placed the patient at undue risk by participating in the study; parasites in the patient's stools, positive <i>Clostridium difficile</i> toxin test and active fungal or viral infection	Group 1: Capsules containing 250 mg leave and stem powder of <i>Artemisia absinthium</i> . [n = 10 (male/female: 6/4)]. 3 caps <i>tids</i> Group 2: No medication. [n = 10 (male/female: 3/7)]	Azathioprine, mesalazine	6 wk	Response: a decrease in the CDAI score of at least 70 points from the qualifying score, or a decrease in 30% of CDAI score from the baseline score
Madsch <i>et al</i> <sup>[20]</sup>	<i>Boswellia serrata</i>	Randomized, placebo-controlled, double-blind	Groups of four patients according to a central computer-generated randomization list	Double-blind	5 patients in <i>Boswellia</i> group	5	Patients, aged between 18 and 80 yr were eligible for the study if they had at least five liquid or soft stools per day on average per week, a complete colonoscopy performed within the last 4 wk before randomization, and a histologically confirmed diagnosis of collagenous colitis	Treatment with budesonide, salicylates, steroids, prokinetics, antibiotics, ketoconazole, or non-steroidal anti-inflammatory drugs within 4 wk before randomization, other endoscopically or histologically verified causes for diarrhea, infectious diarrhea, pregnancy or lactation, previous colonic surgery, and known intolerance to <i>Boswellia</i> extract	Group 1: 400 mg capsules containing <i>Boswellia serrata</i> extract standardized to 80% boswellic acids, 1 capsule <i>tid</i> Group 2: Identical placebo capsules, 1 capsule <i>tid</i>	Loperamide was allowed for the first 3 wk but was not allowed for the last 3 wk of the study. Patients were allowed to use butylscopolamine in case of abdominal pain	6 wk	Clinical remission (stool frequency equal to or less than three soft or solid stools per day on average during the last week of treatment)
Omer <i>et al</i> <sup>[9]</sup>	<i>Artemisia absinthium</i>	Double-blind, placebo-controlled	ND	Double-blind	ND	2	Patients between 18 and 80 yr with CDAI $\geq$ 200 at least for 3 mo receiving CD treatments with 5-aminosalicylates stable dose for at least 4 wk, azathioprine stable dose for 8 wk, methotrexate stable dose for 6 wk or corticosteroids (prednisolone, prednisone or budesonide) at the equivalent of 40 mg/d of prednisone or Less stable dose for 3 wk	Treatment with infliximab; patients with serious pathological findings in ECG, liver, kidney and heart functions, or coexisting organic diseases such as a history of cancer, asthma or other autoimmune disease, or pregnancy; any condition that in the investigators opinion placed the patient at undue risk by participating in the study; parasites in the patient's stools, positive <i>Clostridium difficile</i> toxin test and active fungal or viral infection	Group 1: Capsules containing 250 mg leave and stem powder of <i>Artemisia absinthium</i> . [n = 20 (male/female: 12/8)]. 3 caps <i>bid</i> Group 2: The same capsules without <i>Artemisia absinthium</i> . [n = 20 (male/female: 11/9)]. 3 caps <i>bid</i>	Glucocorticoids, 5-aminosalicylates, azathioprine, methotrexate	10 wk	A decrease in the CDAI score of at least 70 points from the qualifying score, or a decrease in 30% of CDAI score from the baseline score

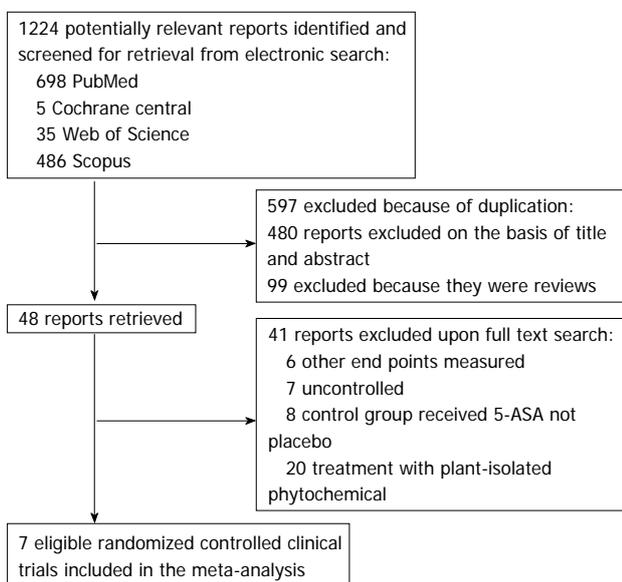
Langmead <i>Aloe vera</i> <i>et al.</i> <sup>[6]</sup>	Randomized, double-blind, placebo-controlled in 2:1 ratio. Computer-generated, block-design.	Double-blind	6 patients in aloë group and 3 in the placebo group	4	Age of 18-80 yr. mildly to moderately active UC (as defined by a modified SCCAI $\geq 3$ ) and no recent changes in conventional prophylactic therapy	Acute severe UC requiring hospital admission (SCCAI > 12); inactive disease (SCCAI < 3); positive stool examination for pathogens; CD or indeterminate colitis; use of antibiotics, warfarin, cholestyramine, sucralfate, anti-diarrhoeal drugs (loperamide, codeine phosphate, diphenoxylate), non-steroidal anti-inflammatory drugs, aspirin > 75 mg/d, aloë vera or other herbal remedies; alcohol or drug abuse; pregnancy or breast feeding; female of child-bearing age not taking adequate contraception; participation in another drug trial in the previous 3 mo; and serious liver, renal, cardiac, respiratory, endocrine, neurological or psychiatric illness, alteration in their dosage of aminosaliculates in the previous 4 wk, had taken > 10 mg/d or had altered oral prednisolone dosage in the previous 4 wk, changed their dose of azathioprine or 6-mercaptopurine in the previous 3 mo, or had used more than five corticosteroid or aminosaliculate enemas in the previous 2 wk	Group 1: <i>Aloe vera</i> gel. [n = 30 (male/female: 16/14)]. 100 mL <i>bid</i> Group 2: The same lipiquid product without <i>Aloe vera</i> gel. [n = 14 (male/female: 6/8)]	5-ASA, prednisolone, azathioprine, topical 5-ASA, topical steroid	4 wk	(1) Clinical remission (SCCAI $\leq 2$ ); (2) Sigmoidoscopic remission [Baron score of zero (normal-looking mucosa) or one (mucosal oedema as indicated by loss of the normal vascular pattern)]; (3) Histological remission (Savery-muttu score of $\leq 1$ , <i>i.e.</i> , no loss of colonocytes, absence of crypt inflammation, and normal lamina propria content of mononuclear cells and neutrophils); (4) Clinical improvement (a reduction in SCCAI of $\geq 3$ points); (5) Clinical response (remission or improvement); (6) Sigmoidoscopic improvement (decrease in Baron score of $\geq 2$ points; and (7) Histological improvement (decrease in Savery-muttu score of $\geq 3$ points)
Ben-Arye <i>Triticum aestivum</i> <i>et al.</i> <sup>[23]</sup>	Randomized, double-blind, placebo-controlled	Double blind; both the true juice and the placebo were packaged in coded, identical, sealed, opaque containers. A driver, blinded to the allocation scheme and given only the addresses for each package, then distributed all the packages	2 patients in triticum group and 1 in the placebo group	4	Age > 18 yr; sigmoidoscopic finding of active UC that involves the left colon; clinical activity comparable with UC; no change in drug treatment (type and dosage) in the month prior to entry; lack of serious systemic involvement-fever > 38 °C, erythema nodosum, arthritis; blood hemoglobin > 11 g%; negative stool culture and test for ova and parasites	ND	Group 1: 100 mL of <i>Triticum aestivum</i> seed juice. [n = 11 (male/female: 6/5)] Group 2: 100 mL of matching placebo. [n = 12 (male/female: 9/3)]	-	1 mo	Improvement (larger than 0.4 in an analog scale where -3 designates the lowest score of aggravation, 0 no change, and +3 highest score of improvement)

CD: Crohn's disease; CDAI: Crohn's disease activity index; ND: Not determined; SCCAI: Simple clinical colitis activity index; ASA: Aminosalicic acid. ECC: Electrocardiography; TNF: Tumor necrosis factor.

**Table 2 Results for outcomes investigated for each included studies**

Herbal product	IBD type	Study	Patients reported AE		Clinical efficacy		Endoscopic efficacy		Histological efficacy		Recurrence relapse
			Any AE	Serious AE	Clinical remission	Clinical response	Endoscopic remission	Endoscopic response	Histological remission	Histological response	
<i>Aloe vera</i>	UC	16	H: 6/30 C: 4/14	-	H: 9/30 C: 1/14	H: 14/30 C: 2/14	H: 7/26 C: 2/11	H: 12/26 C: 3/11	H: 6/21 C: 4/9	H: 14/21 C: 7/9	-
<i>Andrographis paniculata</i>	UC	17	H: 84/149 C: 45/75	H: 4/149 C: 2/75	H: 53/148 C: 19/75	H: 78/148 C: 30/75	H: 65/148 C: 25/75	-	-	-	-
<i>Artemisia absinthium</i>	CD	18	-	H: 0/10 C: 0/10	-	H: 8/10 C: 2/10	-	-	-	-	-
<i>Artemisia absinthium</i>	CD	19	-	-	H: 13/20 C: 0/20	H: 18/20 C: 0/20	-	-	-	-	-
<i>Boswellia serrata</i>	CD	20	H: 29/42 C: 34/40	H: 4/42 C: 4/40	-	-	-	-	-	-	H: 14/42 C: 14/40
<i>Boswellia serrata</i>	Collagenous colitis	21	H: 2/16 C: 1/15	H: 0/16 C: 0/15	H: 10/16 C: 4/15	-	-	-	-	-	-
<i>Triticum aestivum</i>	UC	22	-	-	-	H: 10/11 C: 5/12	-	-	-	-	-

AE: Adverse event; C: Control; CD: Crohn’s disease; H: Herbal product; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.



**Figure 1** Flow diagram of the study selection process. ASA: Aminosallylic acid.

and 10 wk<sup>[16-19,21,22]</sup>. Maintenance of remission was evaluated in one study and duration of this study was 52 wk<sup>[20]</sup>. Scientific name of plant(s) used in herbal medicine, study design, inclusion and exclusion criteria, interventions, concomitant medications, patients’ characteristics, duration of study and definition of outcomes investigated in each included study have been shown in Table 1. Results of investigated outcomes for each included study have been demonstrated in Table 2.

**Efficacy**

**Clinical remission:** The summary for RR of clinical remission in IBD patients for four included trials comparing herbal medicines to placebo<sup>[16,17,19,21]</sup> was 2.07 with 95%CI: 1.41-3.03 ( $P = 0.0002$ , Figure 2A). The Cochrane  $Q$  test for heterogeneity indicated that the studies are not

heterogeneous ( $P = 0.08$ , Figure 2B) and could be combined, thus fixed effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical remission in IBD patients among herbal medicines *vs* placebo therapy was 2.02 (95%CI: 0.37-3.67,  $P = 0.03$  and Kendall’s tau = 1,  $P = 0.08$  (Figure 2C).

The RR of clinical remission in patients with CD<sup>[19]</sup> was 27 with 95%CI: 3.23-260.81, a significant RR.

The summary for RR of clinical remission in UC patients for two included trials comparing herbal medicines to placebo<sup>[16,17]</sup> was 1.59 with 95%CI: 0.8-3.15 ( $P = 0.18$ , Figure 3A). The Cochrane  $Q$  test for heterogeneity indicated that the studies are not heterogeneous ( $P = 0.28$ , Figure 3B) and could be combined but because of few included studies random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical remission in UC patients could not be calculated because of too few strata.

Based on plant type, RR of clinical remission was significant for *Artemisia absinthium* (27.00; 95%CI: 3.23-260.81) and *Boswellia serrata* (2.34; 95%CI: 1.02-6.07) and non-significant for *Aloe vera* and *Andrographis paniculata* (Table 3).

**Clinical response:** The summary for RR of clinical response in IBD patients for five included trials comparing herbal medicines to placebo<sup>[16-19,22]</sup> was 2.59 with 95%CI: 1.24-5.42 ( $P = 0.01$ , Figure 4A). The Cochrane  $Q$  test for heterogeneity indicated that the studies are heterogeneous ( $P = 0.08$ , Figure 4B) and could not be combined, thus the random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response in IBD patients was 2.33 (95%CI: 1.55-3.11,  $P = 0.003$ ) and Kendall’s tau = 0.8,  $P = 0.08$  (Figure 4C).

The summary for RR of clinical response in CD patients for two included trials<sup>[18,19]</sup> was 9.61 with 95%CI:

**Table 3 Results obtained from sub-analyses based on plant type**

Plant	IBD type	Study	Patients reported AE		Clinical efficacy		Endoscopic efficacy		Histological efficacy		Recurrence relapse
			Any AE	Serious AE	Clinical remission	Clinical response	Endoscopic remission	Endoscopic response	Histological remission	Histological response	
<i>Aloe vera</i>	UC	16	0.70 (0.25-2.08)	-	4.20 (0.84-24.84)	3.27 (1.06-12.13)	1.48 (0.44-5.84)	1.69 (0.69-5.04)	0.64 (0.25-1.81)	0.86 (0.55-1.55)	-
<i>Andrographis paniculata</i>	UC	17	0.94 (0.75-1.20)	1.01 (0.22-4.65)	1.41 (0.92-2.23)	1.32 (0.98-1.84)	1.32 (0.93-1.93)	-	-	-	-
<i>Artemisia absinthium</i>	CD	18	-	1.00 (0.06-16.69)	-	9.61 (0.73-126.15), <i>P</i> = 0.09	-	-	-	-	-
<i>Boswellia serrata</i>	CD	19	-	-	27.00 (3.23-260.81)	-	-	-	-	-	-
	CD	20	0.82 (0.66-1.04), <i>P</i> = 0.11	0.95 (0.27-3.31), <i>P</i> = 0.94	-	-	-	-	-	-	0.95 (0.52-1.73)
	Collage-nous colitis	21	-	-	2.34 (1.02-6.07)	-	-	-	-	-	-
<i>Triticum aestivum</i>	UC	22	-	-	-	2.18 (1.19-4.78)	-	-	-	-	-

Results are expressed as relative risk (95%CI). AE: Adverse event; CD: Crohn’s disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

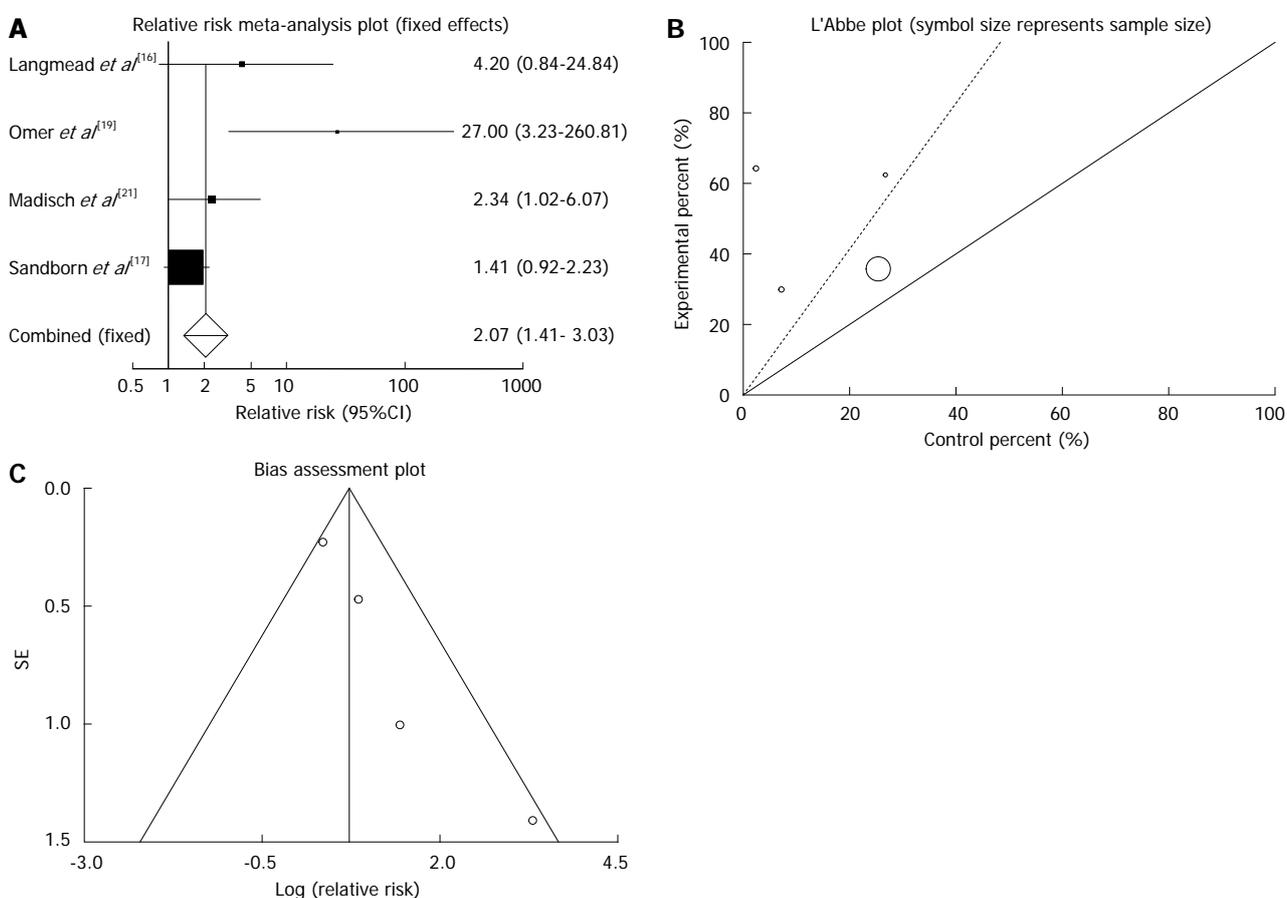


Figure 2 Individual and pooled relative risk (A), heterogeneity indicators (B) and publication bias indicators (C) for the outcome of “clinical remission” in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

0.73-126.15 (*P* = 0.09, Figure 5A). The Cochrane *Q* test for heterogeneity indicated that the studies are not heterogeneous (*P* = 0.08, Figure 5B) and could be combined but because of few included studies the random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response in CD patients could not be calculated because of too few strata.

The summary for RR of clinical response in UC

patients for three included trials comparing herbal medicines to placebo<sup>[16,17,22]</sup> was 1.67 with 95%CI: 1.06-2.65 (*P* = 0.03, Figure 6A). The Cochrane *Q* test for heterogeneity indicated that the studies are not heterogeneous (*P* = 0.22, Figure 6B) and could be combined but because of few included studies the random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response in UC patients among herbal medicines *vs* pla-

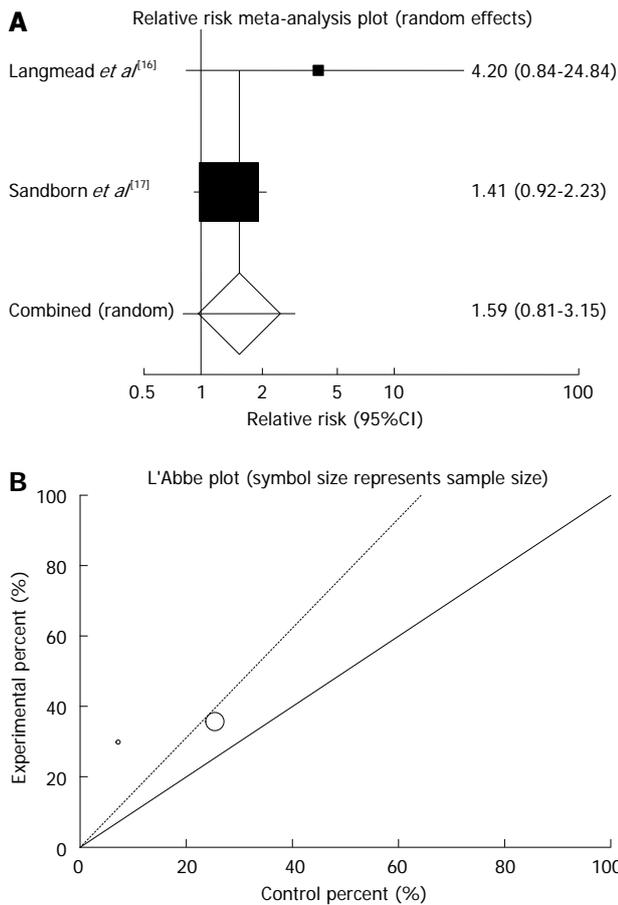


Figure 3 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of “clinical remission” in the studies considering herbal medicines comparing to placebo therapy in ulcerative colitis patients.

cebo therapy could not be calculated because of too few strata.

Based on plant type, RR of clinical response was significant for *Aloe vera* (3.27; 95%CI: 1.06-12.13) and *Triticum aestivum* (2.18; 95%CI: 1.19-4.78) and non-significant for *Andrographis paniculata* and *Artemisia absinthium* (Table 3).

**Endoscopic remission:** The summary for RR of endoscopic remission in IBD patients for two included trials (all of the patients in these studies had UC) comparing herbal medicines to placebo<sup>[16,17]</sup> was 1.33 with 95%CI: 0.93-1.9 ( $P = 0.12$ , Figure 7A). The Cochrane  $Q$  test for heterogeneity indicated that the studies are not heterogeneous ( $P = 0.87$ , Figure 7B) and could be combined but because of few included studies random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for endoscopic remission in IBD (UC) patients could not be calculated because of too few strata.

Based on plant type, RR of endoscopic remission was non-significant for *Aloe vera* (1.48; 95%CI: 0.44-5.84) and *Andrographis paniculata* (1.32; 95%CI: 0.93-1.93) (Table 3).

**Endoscopic response:** The RR of endoscopic response in UC patients comparing herbal medicines with pla-

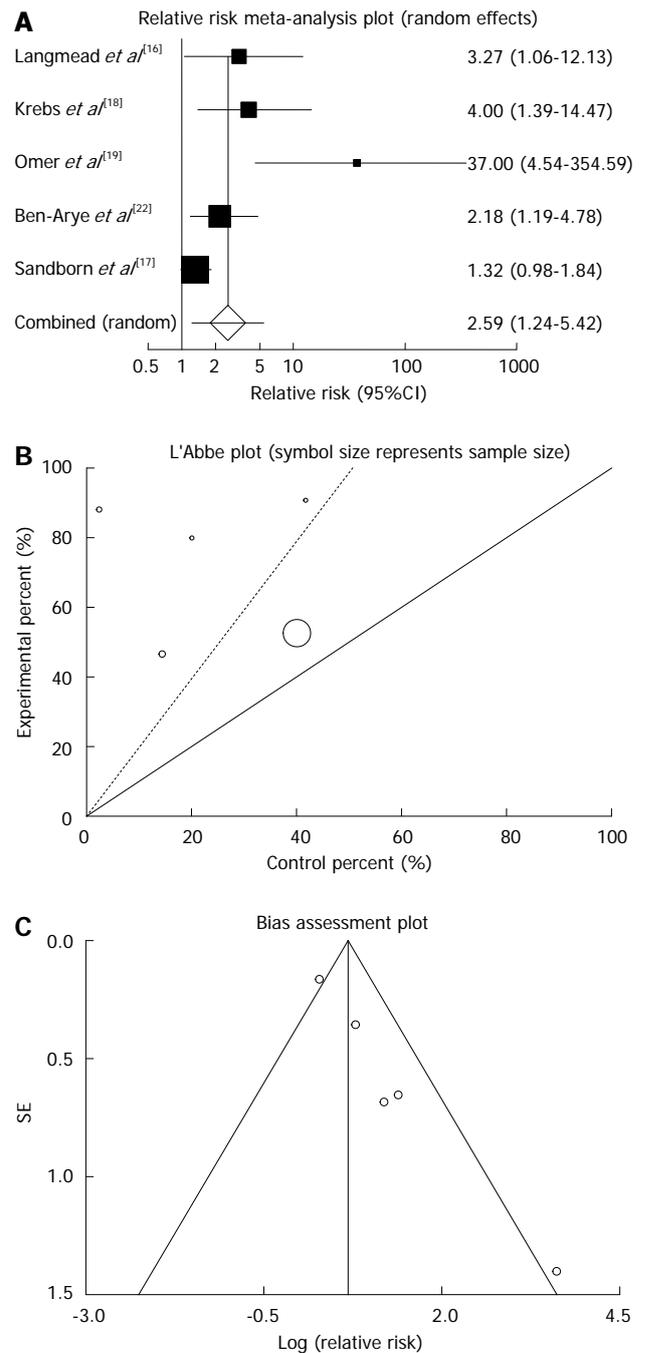


Figure 4 Individual and pooled relative risk (A), heterogeneity indicators (B) and publication bias indicators (C) for the outcome of “clinical response” in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

cebo<sup>[16]</sup> was 1.69 with 95%CI: 0.69-5.04, a non-significant RR.

**Histological remission:** The RR of histological remission in IBD (UC) patients comparing herbal medicines with placebo<sup>[16]</sup> was 0.64 with 95%CI: 0.25-1.81, a non-significant RR.

**Histological response:** The RR of histological response in UC patients comparing herbal medicines with placebo<sup>[16]</sup> was 0.86 with 95%CI: 0.55-1.55, a non-significant RR.

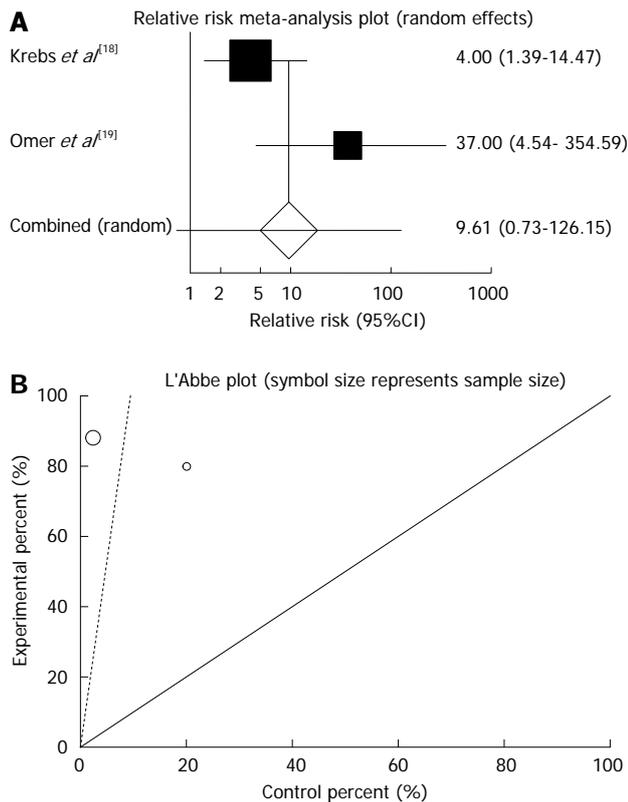


Figure 5 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of “clinical response” in the studies considering herbal medicines comparing to placebo therapy in Crohn’s disease patients.

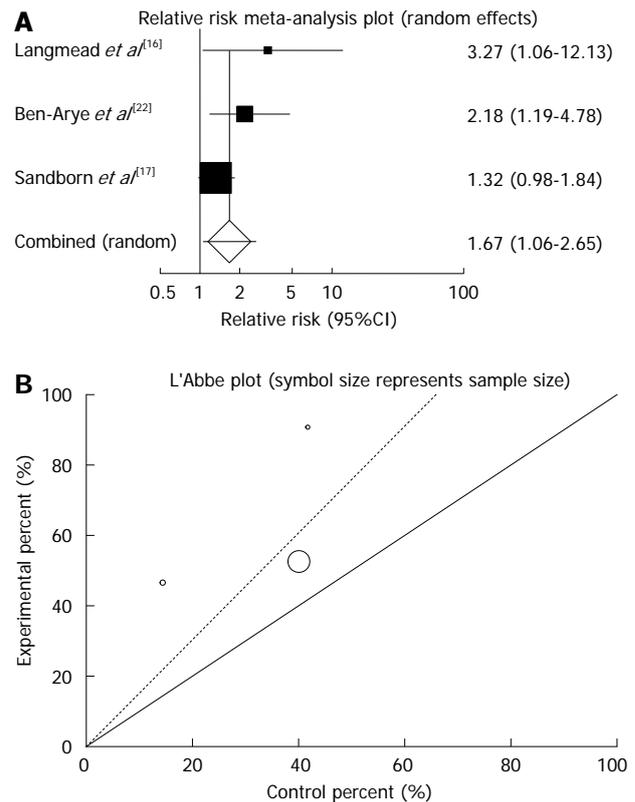


Figure 6 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of “clinical response” in the studies considering herbal medicines comparing to placebo therapy in ulcerative colitis patients.

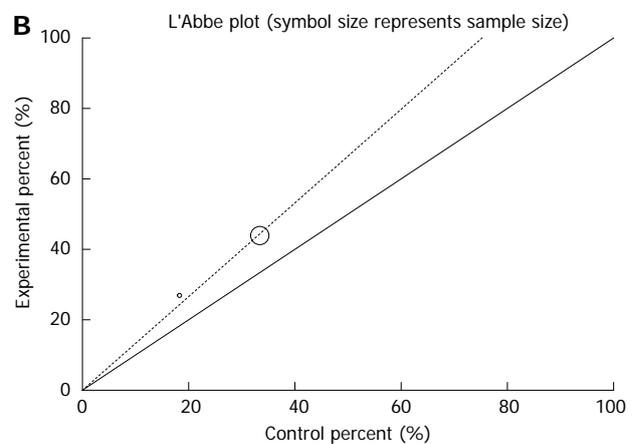
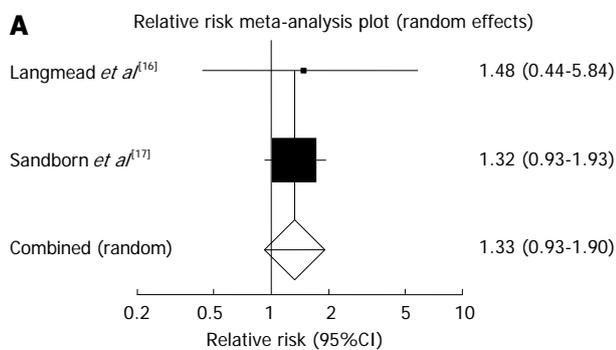


Figure 7 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of “endoscopic remission” in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease (ulcerative colitis) patients.

**Relapse:** The RR of relapse in CD patients comparing herbal medicines with placebo<sup>[20]</sup> was 0.95 with 95%CI: 0.52-1.73, a non-significant RR.

**Tolerability**

**Any adverse events:** The summary for relative risk (RR) of any adverse events in IBD patients for four included trials comparing herbal medicines to placebo<sup>[16,17,20,21]</sup> was 0.89 with 95%CI: 0.75-1.06 ( $P = 0.2$ , Figure 8A). The Cochrane Q test for heterogeneity indicated that the studies

are not heterogeneous ( $P = 0.71$ , Figure 8B) and could be combined, thus fixed effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for any adverse events in IBD patients was 0.18 (95%CI: -2.73-3.09,  $P = 0.81$ ) and Kendall’s tau = 0,  $P = 0.75$  (Figure 8C).

**Serious adverse events:** The summary for RR of serious adverse events in IBD patients for four included trials comparing herbal medicines to placebo<sup>[17,18,20,21]</sup> was

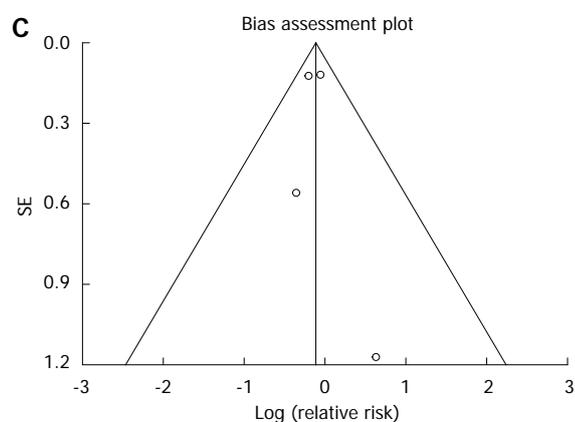
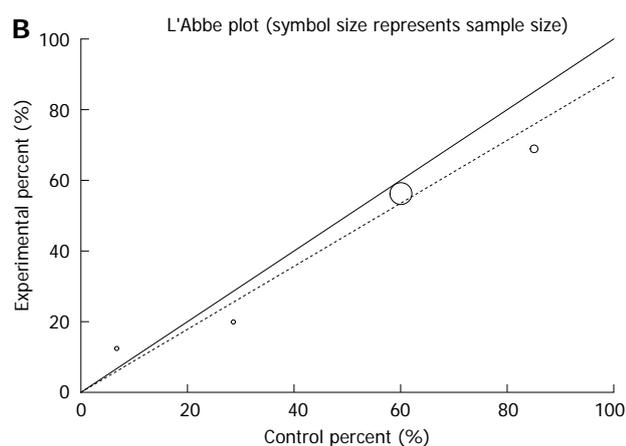
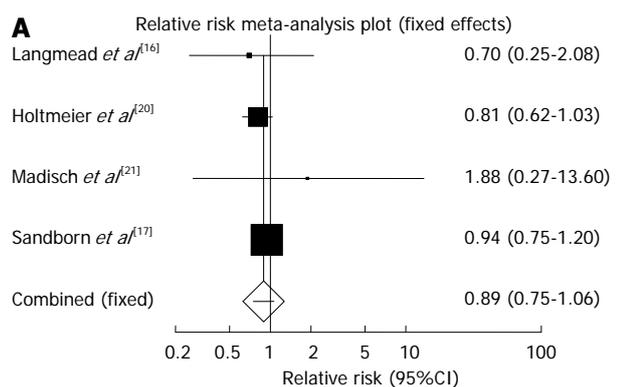


Figure 8 Individual and pooled relative risk (A), heterogeneity indicators (B) and publication bias indicators (C) for the outcome of “any adverse events” in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

0.97 with 95%CI: 0.37-2.56 ( $P = 0.96$ , Figure 9A). The Cochrane  $Q$  test for heterogeneity indicated that the studies are not heterogeneous ( $P > 0.99$ , Figure 9B) and could be combined, thus fixed effects for individual and summary of RR was applied. Regression of normalized effect  $\nu$ s precision for all included studies for serious adverse events in IBD patients was 0.01 (95%CI: -0.19-0.21,  $P = 0.83$ ) and Kendall's tau = 0,  $P = 0.75$  (Figure 9C).

## DISCUSSION

In the current meta-analysis, the efficacy and tolerability

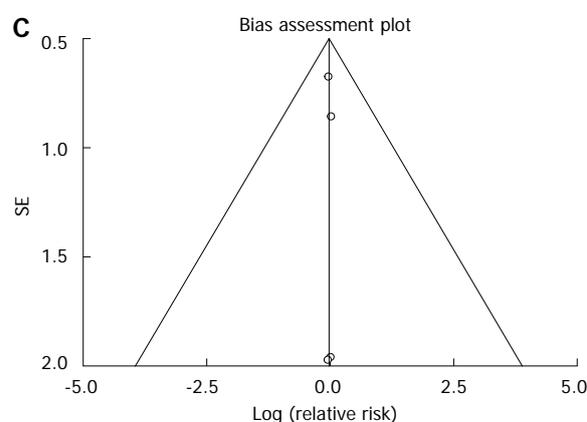
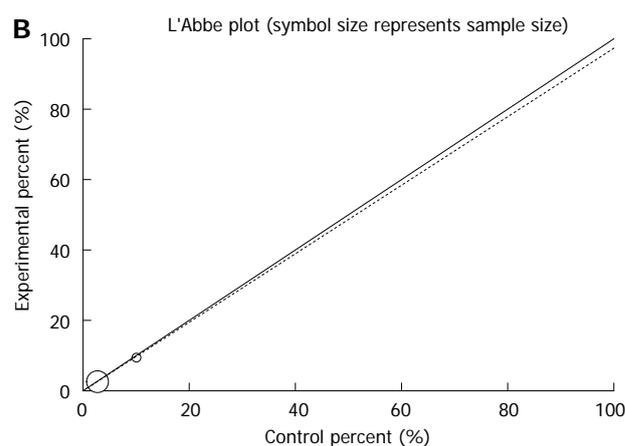
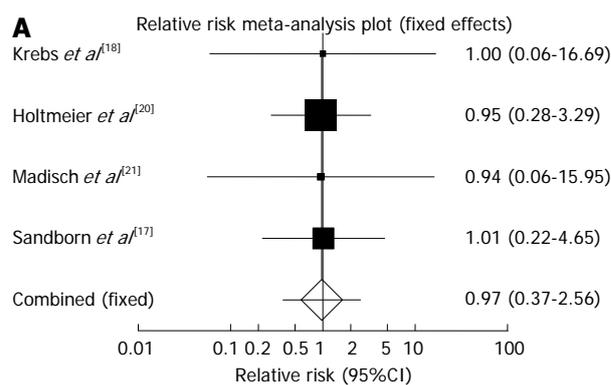


Figure 9 Individual and pooled relative risk (A), Heterogeneity indicators (B) and publication bias indicators (C) for the outcome of “serious adverse events” in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

of herbal medicines in the management all forms of IBD were compared with placebo. The results showed that herbal medicines may induce clinical remission and clinical response in patients with IBD. Endoscopic efficacy was investigated in two studies, both on patient with UC. Herbal medicines did not demonstrate significant effect on induction of endoscopic remission and endoscopic response. Histopathological efficacy was also evaluated in two studies both on patients with UC and the results were the same as endoscopic efficacy. This may be due to short duration of studies and possible slow action of herbal medicines. Moreover, the scoring system used to

assess the mucosal appearance macroscopically is prone to inter-observer variability resulting in non-detecting a significant improvement<sup>[23]</sup>.

The efficacy of herbal medicines in prevention of relapse was investigated in only one study and showed no priority of these products compared to placebo. The number of patients showed any adverse events or serious adverse events were not significantly different between herbal medicines and placebo and this confirmed safety and tolerability of these products.

The present meta-analysis may have been limited by small sample sizes of studies and heterogeneity. Since the included trials involved herbal medicines contained different plants administered to patients with various subtypes of IBD, the trials were disaggregated. Thus, sub-analyses based on type of IBD and plant type was performed. The results of sub-analysis based on IBD type showed that herbal medicines significantly induce clinical remission in patients with CD and clinical response in patients with UC; however the induction of clinical remission in patients with UC and induction of clinical response in patients with CD by herbal medicines were not significant. The results of sub-analyses based on plant type demonstrated that induction of clinical remission was obtained only by *Artemisia absinthium* and *Boswellia serrata* and induction of clinical response was gained by only *Aloe vera* and *Triticum Aestivum*. None of the plants caused induction of endoscopic or histological efficacy. *Boswellia serrata* in one study evaluating recurrence rate did not cause prevention of relapse. Induction of adverse events by none of the plants was significant in comparison to that of placebo.

Overall, the results show that herbal medicines may induce clinical efficacy in patients with IBD, but the evidence is too limited to make any confident conclusions. Meta-analysis of clinical trials that have compared efficacy of herbal medicines with that of conventional drugs such as amino-salicylates can be helpful that is being carried out by authors of this paper. Further high quality, large controlled trials using standardized preparation are warranted to better elucidate the effects of these herbs in IBD.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of gastrointestinal tract. Due to lack of desired efficacy and poor tolerability of conventional drugs, approach toward complementary and alternative medicines especially herbal medicines for the management of IBD are growing. Besides many experimental studies, the efficacy and tolerability of herbal medicines in IBD have been investigated through several clinical trials.

### Research frontiers

Although the efficacy and tolerability of herbal medicines for the management of IBD were evaluated through several clinical trials in comparison to placebo, no meta-analysis has been conducted to reach a convincing conclusion.

## Innovations and breakthroughs

Based on this meta-analysis, herbal medicines may safely induce clinical response and remission in patients with IBD without significant effects on endoscopic and histological outcomes, but the number of studies is yet limited to make a strong conclusion.

## Applications

Regarding desirable effects of herbal medicine in induction of clinical response and remission in IBD and their low adverse events, it would not be surprising to introduce good medicines to clinic if proper standardization and dose adjustments are done.

## Peer review

The aim of the study was to evaluate the efficacy and tolerability of herbal medicines in IBD by conducting a meta-analysis. This paper is good for IBD community.

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**S- Editor** Wen LL **L- Editor** A **E- Editor** Li JY



## Pancreatic paracoccidioidomycosis simulating malignant neoplasia: Case report

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(US) showed a solid mass of approximately 7 cm × 5.5 cm on the pancreas head. Abdominal computerized tomography showed dilation of the biliary tract, an enlarged pancreas (up to 4.5 in the head region), with dilation of the major pancreatic duct. The patient underwent exploratory laparotomy, and the surgical description consisted of a tumor, measuring 7 to 8 cm with a poorly-defined margin, adhering to posterior planes and mesenteric vessels, showing an enlarged bile duct. External drainage of the biliary tract, Roux-en-Y gastroenteroanastomosis, lymph node excision, and biopsies were performed, but malignant neoplasia was not found. Microscopic analysis showed chronic pancreatitis and a granulomatous chronic inflammatory process in the choledochal lymph node. Acid-alcohol resistant bacillus and fungus screening were negative. Fine-needle aspiration of the pancreas was performed under US guidance. The smear was compatible with infection by *Paracoccidioides brasiliensis*. We report a rare case of paracoccidioidomycosis simulating a malignant neoplasia in the pancreas head.

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**Key words:** Paracoccidioidomycosis; Pancreas; Fungus infection; Pancreatic tumors; Differential diagnosis

### Abstract

Paracoccidioidomycosis is a systemic granulomatous disease caused by fungus, and must be considered in the differential diagnosis of intra-abdominal tumors in endemic areas. We report a rare case of paracoccidioidomycosis in the pancreas. A 45-year-old man was referred to our institution with a 2-mo history of epigastric abdominal pain that was not diet-related, with night sweating, inappetence, weight loss, jaundice, pruritus, choluria, and acholic feces, without signs of sepsis or palpable tumors. Abdominal ultrasonography

**Core tip:** This is a report of a rare case of pancreatic paracoccidioidomycosis, which shows its importance in the differential diagnosis of intra-abdominal tumors in endemic areas. This is apparently the first such report written in English. The patient had a pancreatic mass adhering to vessels and deep planes, with enlargement of satellite lymph nodes; but malignant neoplasia was not found. The ultrasonography-guided pancreas fine-needle aspiration defined the diagnosis and successfully directed the therapy. Remarkably, although the patient had abdominal lymph node enlargement, he did not present peripheral lymphadenopathy, which is usually

the major complaint in patients with the juvenile form of paracoccidioidomycosis.

Lima TB, Domingues MAC, Caramori CA, Silva GF, Oliveira CV, Yamashiro FS, Franzoni LC, Sasaki LY, Romeiro FG. Pancreatic paracoccidioidomycosis simulating malignant neoplasia: Case report. *World J Gastroenterol* 2013; 19(34): 5750-5753 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5750.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5750>

## INTRODUCTION

Paracoccidioidomycosis (also known as Pb mycosis or South American blastomycosis) is a systemic granulomatous disease caused by the fungus *Paracoccidioides brasiliensis*<sup>[1]</sup>, which is autochthonous in Latin America. It was described by Lutz<sup>[2]</sup>, and its incidence ranges from 3 to 4 cases per million, up to 1 to 3 cases per one hundred thousand inhabitants per year in endemic areas, with an annual mortality of 1.45 per million inhabitants, which is the highest rate observed among systemic mycoses<sup>[1]</sup>. It affects 10 to 15 adult males per one female case, mostly between the 3<sup>rd</sup> and 6<sup>th</sup> decades of life, and has two main forms: the juvenile form, constituting 3%-5% of cases, which compromises the reticuloendothelial system, and the somewhat more common chronic form that comprises 90% of cases, mainly affecting the skin and the lungs<sup>[1]</sup>. Paracoccidioidomycosis can mimic neoplasias, such as periampullary<sup>[3]</sup> or colon<sup>[4]</sup> cancer. Therefore, it must be considered in the differential diagnosis of intra-abdominal tumors in endemic areas<sup>[5]</sup>. This case illustrates a rare case of paracoccidioidomycosis simulating a malignant neoplasia in the pancreas head.

## CASE REPORT

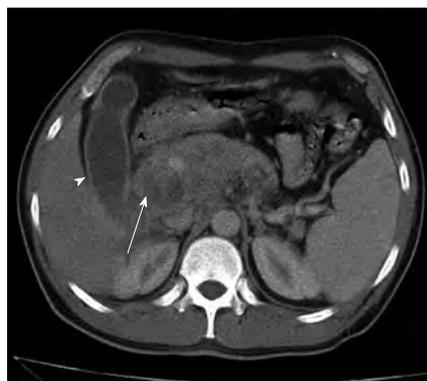
A 45-year-old white adult male from São Paulo state was referred to our institution with a 2-mo history of epigastric pain that was not diet-related. It was intermittent, of strong intensity, with radiation to the back and relieved by the use of omeprazole. Additionally, the patient had night sweating and a weight loss of 13 kg in 45 d, and lacked vomiting, diarrhea, or fever. Twenty days before admission, the patient reported jaundice, pruritus over the whole body, choloria, and acholic feces. The patient was a current smoker and alcohol user, while his physical examination revealed jaundice and pain after deep abdominal palpation, without signs of sepsis or palpable tumors. Pulmonary auscultation showed no alterations, and laboratory exams confirmed cholestatic syndrome (Table 1).

Abdominal ultrasonography (US) showed a solid mass of approximately 7 cm × 5.5 cm on the pancreas head, with a small tumor of 1 to 2 cm juxtaposed with the mass, suggesting local metastases. Abdominal com-

**Table 1** Laboratory exams according to the time of hospitalization

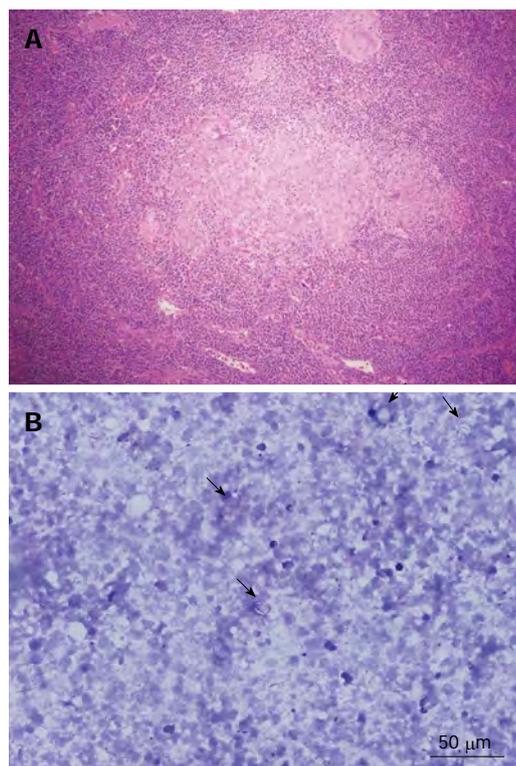
	Admission	3 mo	12 mo	Normal range
GGT (U/L)	646	677	632	15-73
Alkaline phosphatase (U/L)	1047	600	262	36-126
Aspartate transaminase (U/L)	58	147	68	30-110
Alanine transferase (U/L)	53	184	173	21-75
Albumin (g/dL)	2.5	4.3	4.8	3.5-5
INR	1.04	1.17	1.12	< 1.25
Total bilirubin (mg/dL)	3.8	1.7	0.6	0.2-1.3
Indirect bilirubin (mg/dL)	0.6	0.4	0.2	0-1.1
Direct bilirubin (mg/dL)	1.2	0.1	0	0-0.3
Platelets (× 10 <sup>3</sup> /mm <sup>3</sup> )	278	177	138	140-440
Leukocytes (× 10 <sup>3</sup> /mm <sup>3</sup> )	6.2	4.5	5.3	4-11

GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio.



**Figure 1** Contrast enhanced abdominal computed tomography. Head, body, and tail of pancreas with enlarged dimensions and a poorly-defined margin, with two hypodense areas without significant enhancement after intravenous contrast (white arrow). The same image shows a hypodense gallbladder (arrow head) and mild splenomegaly.

puterized tomography (CT) showed dilation of the biliary tract, an enlarged pancreas (up to 4.5 in the region of the head) with a poorly-defined margin and two hypodense areas without significant enhancement after intravenous contrast, leading to dilation of the major pancreatic duct, but without retroperitoneal lymphadenopathy (Figure 1). Due to the momentary unavailability of the necessary equipment at our institution to perform a puncture guided by CT or US, the patient underwent exploratory laparotomy. The surgical description consisted of a pancreatic tumor measuring 7 to 8 cm with a poorly-defined margin, adhering to posterior planes and to mesenteric vessels, and showing an enlarged bile duct. Transcystic cholangiography showed obstruction of the passage of contrast through the distal bile duct, which was compatible with extrinsic compression. External drainage of the biliary tract, Roux-en-Y gastroenteroanastomosis, lymph node excision, and biopsies were performed. Malignant



**Figure 2** Pathological findings (hematoxylin/eosin  $\times$  200 magnification). A: Epithelioid granuloma in a lymph node near the ductus choledochus, with absence of necrosis, fungi, or bacteria; B: Fine-needle aspiration smear showing rounded yeast forms with a birefringent wall and multiple sporulation compatible with *Paracoccidioides brasiliensis* (black arrows).

neoplasia was not found at the intraoperative frozen section analysis. Microscopic analysis showed chronic pancreatitis and a granulomatous chronic inflammatory process in the choledochal lymph node. The presence of granulomas without caseous necrosis in the lymph node would suggest a diagnosis of sarcoidosis; however, at that time it was not possible to definitively discard the diagnosis of tuberculosis (Figure 2A). Acid-alcohol resistant bacillus and fungus screening by Ziehl-Neelsen, PAS, and Gömöri staining were negative. The biopsies were reviewed, and a cohesive granuloma without caseous necrosis was found attached to peripancreatic fat. The abdominal pain and the jaundice worsened, and the patient developed fever, leukocytosis, and drainage of purulent secretion by a surgical drain located near the pancreas. Upper digestive endoscopy showed esophageal varices. Plain chest X-ray and thoracic CT scan were normal. Metronidazole and ciprofloxacin were prescribed due to the hypothesis of bacterial cholangitis, and clinical improvement was observed. Fine-needle aspiration (FNA) of the pancreas was performed with US guidance. The obtained smear was compatible with infection by *Paracoccidioides brasiliensis* (Figure 2B). Enzyme-linked immunosorbent assay tests, employed for the detection of anti-*Paracoccidioides brasiliensis* antibodies, produced positive results (titers of 1/8 and 1/16). Intravenous sulfamethoxazole-trimethoprim (800/160 mg two times per day) was introduced, after which the patient showed

clinical and laboratory improvement (Table 1).

## DISCUSSION

Pancreatic paracoccidioidomycosis simulating pancreatic neoplasia has been infrequently reported in the literature<sup>[6]</sup>. Clinical findings include weight loss, weakness, dizziness, repletion, pruritus, jaundice, choluria, and fecal acholia, as in the reported case. Signs of cholestatic disease are the most reported abnormalities, with an increase in alkaline phosphatase and gamma-glutamyl transferase. Jaundice is usually observed in the late stage of the disease, and is associated with differential diagnoses of greater severity<sup>[7]</sup>, such as malignant pancreatic neoplasia. The findings of pancreatic tumor adhering to vessels and deep planes, as well as satellite nodules and obstruction of the biliary tract with secondary portal hypertension (diagnosed through the esophageal varices), suggested a diagnosis of metastatic pancreatic cancer. As shown in this report, abdominal lymphatic compromise may give rise to such clinical conditions as abdominal tension and pain, which may even simulate acute abdomen affections<sup>[8]</sup>. The granulomatous involvement of lymph nodes initially led to the hypothesis of sarcoidosis, which may affect multiple organs, particularly the lungs (90%) and lymph nodes (75%)<sup>[9]</sup>. But sarcoidosis rarely affects the pancreas<sup>[10,11]</sup>, and when it does, it is usually asymptomatic<sup>[12]</sup>. The type of granuloma found in the lymph node, characteristically epithelioid, without necrosis or bacilli, favored this diagnosis. On the other hand, immunosuppression with corticoids, which would be recommended in a case of sarcoidosis, may be harmful if the patient has had an infectious disease. At that time, the second pancreatic tissue sample obtained by FNA defined the paracoccidioidomycosis diagnosis and successfully directed the therapy. Interestingly, although the patient had had an abdominal lymph node enlargement, he did not have peripheral lymphadenopathy, which is usually the major complaint in patients with the juvenile form of paracoccidioidomycosis. Additionally, there were none of the pulmonary manifestations that occur in 90% of patients with the chronic form<sup>[1]</sup>. Although it is rare, pancreatic paracoccidioidomycosis must be considered in the differential diagnosis of intra-abdominal tumors in endemic areas, even without peripheral lymphadenopathy.

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## Extended therapy duration for therapy-refractory hepatitis C patients with genotype 2

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### Abstract

We devised an extended 72-wk peginterferon- $\alpha$ -2a/ribavirin therapy regimen for the retreatment of highly intractable cases, *i.e.*, 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy-intractable cases. Although 2 cases achieved a rapid virological response to 72-wk peginterferon- $\alpha$ -2a/ribavirin therapy, 1 case failed to achieve a sustained virological response. Although the reason for this difference in the effectiveness of 72-wk peginterferon- $\alpha$ -2a/ribavirin therapy between the cases was unclear, the rebound phenomenon of serum transaminase after 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy and the resultant lower viral load compared to that before 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy might have influenced the treatment outcome. Thus, it may be beneficial to consider the rebound phenomenon of serum transaminase and the changes in viral load resulting from previous interferon-based therapy and then cau-

tiously determine the indication and the timing of the administration of 72-wk peginterferon- $\alpha$ -2a/ribavirin in highly intractable cases. Further studies should be performed to confirm this strategy.

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**Key words:** Hepatitis C; Genotype 2 and high viral loads; Interferon-based therapy; Highly intractable case; Extended therapy duration

**Core tip:** The optimal therapy for 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy-intractable hepatitis C patients with genotype 2 and high viral loads remains unknown. Our cases are notable in that 72-wk peginterferon- $\alpha$ -2a/ribavirin therapy may have been effective for these highly intractable cases. Additionally, the rebound phenomenon of serum transaminase after the 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy and the resultant lower viral load compared to that before the 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy might have influenced the treatment outcome. Thus, our cases highlight the importance of the results of the previous 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy in the indication and timing of the administration of 72-wk peginterferon- $\alpha$ -2a/ribavirin in highly intractable cases.

Sato K, Yanagisawa M, Hashizume H, Yamazaki Y, Horiguchi N, Kakizaki S, Mori M. Extended therapy duration for therapy-refractory hepatitis C patients with genotype 2. *World J Gastroenterol* 2013; 19(34): 5754-5758 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5754.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5754>

### INTRODUCTION

The American Association for the Study of Liver Diseases Practice Guidelines recommend treatment with

peginterferon/ribavirin for 24 wk using a ribavirin dose of 800 mg for interferon-based therapy-naïve patients with hepatitis C virus (HCV) genotype 2<sup>[1]</sup>. However, retreatment with peginterferon/ribavirin in patients who do not achieve a sustained virological response (SVR) after a full course of peginterferon/ribavirin is not recommended, even if a different type of peginterferon is administered<sup>[1]</sup>. However, some patients who have previously completed peginterferon- $\alpha$ -2b [PegIntron; Merck Sharp & Dohme (MSD), Tokyo, Japan]/ribavirin (Rebetol; MSD) therapy but failed to achieve an SVR can be treated with a 24- to 48-wk course of peginterferon/ribavirin<sup>[2-4]</sup>. Oze *et al*<sup>[2]</sup> suggested that the SVR rate was similar between 24-wk and 48-wk peginterferon/ribavirin therapy in patients with genotype 2 who achieved a rapid virological response (RVR) upon retreatment. We previously demonstrated the superiority of 48-wk therapy over 24-wk therapy for patients with genotype 2 and high viral loads  $\geq 10^5$  international units (IU)/mL; 5 logIU/mL as determined using the quantitative reverse transcription polymerase chain reaction-based Cobas TaqMan HCV Test (Roche Diagnostics, Tokyo, Japan) who demonstrated serum HCV RNA levels [measured using the Cobas Amplicor Monitor HCV ver. 2.0 Assay (Roche Diagnostics)]  $\geq 50$  IU/mL at week 4 of therapy regardless of the history of interferon-based therapy<sup>[5]</sup>. The Japan Society of Hepatology also recommends a 24- to 48-wk course of peginterferon/ribavirin retreatment following peginterferon/ribavirin treatment in patients with HCV genotype 2 and high viral loads<sup>[6]</sup>. However, the effectiveness of retreatment in highly intractable cases, *i.e.*, 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy-intractable cases, remains unknown. Therefore, we devised a 72-wk peginterferon/ribavirin therapy regimen for the retreatment of highly intractable cases, regardless of the time of disappearance of serum HCV RNA.

As for the type of peginterferon, the superiority of peginterferon- $\alpha$ -2a therapy over peginterferon- $\alpha$ -2b therapy for chronic hepatitis C has not been determined. However, given that peginterferon- $\alpha$ -2a therapy is significantly superior or has a tendency to increase the efficacy of treatment in patients with HCV genotype 2 compared to peginterferon- $\alpha$ -2b therapy according to randomized controlled trials<sup>[7,8]</sup> and a meta-analysis of randomized controlled trials<sup>[9]</sup>, we selected peginterferon- $\alpha$ -2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan)/ribavirin (Copegus; Chugai Pharmaceutical Co., Ltd.) for the retreatment of 48-wk peginterferon  $\alpha$ -2b/ribavirin therapy-intractable cases.

The submitted case reports comply with the Declaration of Helsinki. Informed consent was obtained from the patients.

## CASE REPORT

### Case 1

A 55-year-old male was referred to our hospital because

of chronic HCV infection and abnormal transaminase levels. His medical history included an operation for the treatment of appendicitis at 8 years of age and acute hepatitis with jaundice at 23 years of age after he had acquired a tattoo and abused drugs at 25 years of age. He had neither a history of blood transfusion nor a family history of liver diseases. The transmission of hepatitis C in this case could have been mediated by either the tattoo or drug abuse. His complete blood counts appeared normal, although blood tests showed elevated levels of serum alanine aminotransferase (ALT) (44 IU/mL). The quantitative detection of HCV RNA [real-time polymerase chain reaction (PCR), COBAS TaqMan Test] revealed a level of 6.5 logIU/mL; the HCV genotype was 2b. The interleukin-28B (IL28B) locus single nucleotide polymorphisms (SNPs) previously reported to be associated with therapy outcome, including rs8099917, rs11881222 and rs8103142<sup>[10]</sup>, were all major homozygous. The inosine triphosphatase (ITPA) locus SNP previously reported to be associated with ribavirin-induced hemolytic anemia<sup>[11]</sup> and interferon-induced thrombocytopenia<sup>[12]</sup> in Japanese chronic hepatitis C patients under peginterferon- $\alpha$ /ribavirin therapy (rs1127354) was major homozygous. A liver biopsy obtained prior to interferon-based therapy was graded F1/A2 according to the New Inuyama classification.

We initiated treatment with peginterferon- $\alpha$ -2b (100  $\mu$ g once weekly, subcutaneously) and ribavirin (800 mg per day) based on the patient's body weight. Serum HCV RNA was not detectable at wk 8 of therapy. Thus, we extended the duration of therapy from the standard 24-wk regimen to a 48-wk regimen based on our prospective study<sup>[5]</sup>. The patient's adherence to peginterferon- $\alpha$ -2b/ribavirin was 100%, although his serum HCV RNA level became positive 4 wk after the completion of therapy. Notably, the rebound phenomenon of serum transaminase after completion of the 48-wk therapy occurred, and the viral load decreased below the pre-treatment level. He was retreated with peginterferon- $\alpha$ -2a (180  $\mu$ g once weekly, subcutaneously) and ribavirin (800 mg per day) based on his body weight. Because the 48-wk therapy with peginterferon- $\alpha$ -2b and ribavirin resulted in viral relapse, we extended the duration of therapy from the recommended 24-48 wk to 72 wk, although the serum level of HCV RNA became undetectable after 4 wk of therapy. Although the patient's adherence to ribavirin was 74% due to anemia, he achieved an SVR. The laboratory findings, treatments and outcomes for case 1 are shown in Table 1.

### Case 2

A 55-year-old male was referred to our hospital because of chronic HCV infection. His previous medical history included an operation due to appendicitis at 14 years of age and euthyroid sick syndrome, the onset of which was unclear. He had neither a history of blood transfusion nor a family history of liver disease. Thus, the transmission source of hepatitis C in this case could not be

**Table 1** Laboratory findings at baseline, treatments and outcomes of highly intractable cases

Parameters	Case 1		Case 2	
Age (yr)	55		55	
Sex	Male		Male	
HCV genotype	2b		2b	
Transmission	Tattoo or drug abuse		Unknown	
IL28B SNPs (rs8099917:rs11881222:rs8103142)	T/T:A/A:T/T		T/T:A/A:T/T	
IITPA SNPs	C/C		C/C	
At the start of therapy	First therapy	Second therapy	First therapy	Second therapy
BMI (kg/m <sup>2</sup> )	26	27.5	20.9	19.7
HCV RNA (logIU/mL)	6.5	3.7	7.2	7.5
Liver biopsy	F1/A2	Not performed	F1/A2	Not performed
ALT (IU/L)	36	26	32	40
AST (IU/L)	44	32	32	33
WBC (cells/ $\mu$ L)	4520	3880	3690	4020
Neutrophil (cells/ $\mu$ L)	2650	2420	1450	1950
Hemoglobin (g/dL)	15.7	14.6	11.9	12.9
Platelets (cells/ $\mu$ L)	16.4	15.7	14.5	15.3
Peak ALT after the first therapy (IU/L)	210		33	
Duration between the first therapy and the second therapy (wk)	17		10	
Treatment and outcome	First therapy	Second therapy	First therapy	Second therapy
Peginterferon $\alpha$ dosage ( $\mu$ g)	100	180	100	180
RBV dosage (mg)	800	800	800	800
Week at disappearance of serum HCV RNA	8	4	16	4
Adherence to peginterferon $\alpha$	100%	100%	100%	100%
Adherence to RBV	100%	74%	96%	100%
Weeks of therapy	48	72	48	72
Response	Relapse	SVR	Relapse	Relapse

HCV: Hepatitis C virus; IL28B: Interleukin-28B; SNPs: Single nucleotide polymorphisms; IITPA: Inosine triphosphatase; first therapy: 48-wk peginterferon  $\alpha$ -2b/ribavirin; second therapy: 72-wk peginterferon  $\alpha$ -2a/ribavirin; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; WBC: White blood cell; RBV: Ribavirin; SVR: Sustained virological response.

identified. A complete blood count showed slight anemia, and blood tests showed a slightly high serum ALT level (32 IU/mL). The quantitative detection of HCV RNA (real-time PCR, COBAS TaqMan Test) revealed a level of 7.2 logIU/mL; the HCV genotype was 2b. The IL28B locus SNPs, including rs8099917, rs11881222 and rs8103142, were all major homozygous. The IITPA locus SNP rs1127354 was major homozygous. A liver biopsy obtained prior to interferon-based therapy was graded F1/A2 according to the New Inuyama classification.

We initiated treatment with peginterferon- $\alpha$ -2b (100  $\mu$ g once weekly, subcutaneously) and ribavirin (800 mg per day) based on the patient's body weight. Serum HCV RNA disappeared at week 16 of therapy. Thus, we extended the therapy duration from the standard 24-wk regimen to a 48-wk regimen based on our prospective study<sup>[5]</sup>. Although the patient's adherence to peginterferon- $\alpha$ -2b/ribavirin was 100%, his serum HCV RNA level became positive 4 wk after the completion of therapy. In contrast to case 1, no obvious rebound phenomenon of serum transaminase was observed after completion of the 48-wk therapy, and the viral load returned to the pre-treatment level. He was retreated with peginterferon- $\alpha$ -2a (180  $\mu$ g once weekly, subcutaneously) and ribavirin (800 mg per day) based on his body weight. Because the 48-wk therapy with peginterferon- $\alpha$ -

2b and ribavirin resulted in viral relapse, we extended the duration of therapy from the recommended 24-48 wk to 72 wk, and his serum HCV RNA level became negative at wk 4 of therapy. Although the patient's adherence to both drugs was 100%, his serum HCV RNA level became positive again at 4 wk after the completion of therapy, and he therefore could not achieve an SVR. The laboratory findings, treatments and outcomes of case 2 are shown in Table 1.

## DISCUSSION

The major findings from these case reports are twofold: 72-wk peginterferon- $\alpha$ -2a/ribavirin therapy may represent an effective therapy for 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy-intractable cases, and by contrast, even 72-wk peginterferon- $\alpha$ -2a/ribavirin therapy-intractable HCV infection can occur in patients with genotype 2 and high viral loads. Although the difference in the effectiveness of 72-wk therapy between the 2 cases was not clear, the rebound phenomenon of serum transaminase after the 48-wk therapy and the resultant lower viral load compared to that before the 48-wk therapy might be possible contributing factors that could influence the treatment outcome, based on these case reports and our previous study<sup>[5]</sup>. Case 1 was consistent with a previous

report indicating that patients who showed biochemical relapse after initial interferon therapy had a significantly lower serum HCV RNA level at recovery after ALT relapse compared to before the initial interferon therapy<sup>[13]</sup>. Moreover, our cases showed that an RVR was not sufficient for predicting an SVR, even for 72-wk interferon-based therapy. Because both of our cases carried major homozygous IL28B SNPs, our findings support a limited role for *IL28B* genotypes regarding the virological responses achieved in chronic hepatitis C patients with genotype 2 and high viral loads<sup>[5]</sup>. However, because the number of our highly intractable cases was small, further studies are needed to test this hypothesis.

One possible solution for the treatment of highly intractable cases is telaprevir in combination with peginterferon- $\alpha$ -2b/ribavirin therapy. In fact, this therapy achieved an SVR in a 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy-intractable female patient with genotype 2 and a high viral load in a phase III clinical trial (unpublished data). In addition, direct-acting anti-viral (DAA) combination therapy may overcome this difficulty in highly intractable cases in the near future. However, for cases in which it is difficult to use DAA due to the risk of drug interactions between DAA and medicines administered to treat complications or for patients who are discouraged from waiting for antiviral therapy, such as those who developed hepatocellular carcinoma, 72-wk peginterferon- $\alpha$ -2a/ribavirin therapy may be one strategy for curing highly intractable patients with genotype 2 and high viral loads. For case 1 presented here, 24- to 48-wk peginterferon- $\alpha$ -2a/ribavirin therapy for retreatment may have been effective, although we selected 72-wk therapy to improve the likelihood of an SVR. However, 72-wk therapy is not always sufficient, as demonstrated in case 2 (although he did achieve an RVR). In case 1, the viral load after 48-wk peginterferon- $\alpha$ -2b/ribavirin therapy decreased significantly in comparison to the level prior to treatment, whereas the viral load returned to the pre-treatment level in case 2. We were able to initiate the 72-wk therapy under the condition that the viral load had become lower than that before the 48-wk therapy in case 1. This indicates that it may be a good strategy to consider the rebound phenomenon of serum transaminase and the changes in viral load as a result of previous interferon-based therapy and then cautiously determine the indication and timing of administration of the 72-wk peginterferon- $\alpha$ -2a/ribavirin in highly intractable cases. However, a therapy duration of less than 72 wk should also be considered as a second therapy if the pre-treatment viral load before the second therapy is low (less than 5 logIU/mL) because chronic hepatitis C patients with low viral loads are likely to achieve an SVR in short-term therapy. Further studies should be examined to confirm the strategy of extended therapy duration.

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## Cerebral and splenic infarctions after injection of *N*-butyl-2-cyanoacrylate in esophageal variceal bleeding

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**Key words:** Cerebrum; Esophageal varix; Infarction; *N*-butyl-2-cyanoacrylate; Spleen

**Core tip:** Variceal bleeding is the most serious complication of portal hypertension, and it accounts for approximately one fifth to one third of all deaths in liver cirrhosis patients. Although injection sclerotherapy with *N*-butyl-2-cyanoacrylate provides effective treatment for variceal bleeding, injection of *N*-butyl-2-cyanoacrylate is associated with a variety of complications including systemic embolization. Herein, we report a case of cerebral and splenic infarctions after the injection of *N*-butyl-2-cyanoacrylate to treat esophageal variceal bleeding.

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### Abstract

Variceal bleeding is the most serious complication of portal hypertension, and it accounts for approximately one fifth to one third of all deaths in liver cirrhosis patients. Currently, endoscopic treatment remains the predominant method for the prevention and treatment of variceal bleeding. Endoscopic treatments include band ligation and injection sclerotherapy. Injection sclerotherapy with *N*-butyl-2-cyanoacrylate has been successfully used to treat variceal bleeding. Although injection sclerotherapy with *N*-butyl-2-cyanoacrylate provides effective treatment for variceal bleeding, injection of *N*-butyl-2-cyanoacrylate is associated with a variety of complications, including systemic embolization. Herein, we report a case of cerebral and splenic infarctions after the injection of *N*-butyl-2-cyanoacrylate to treat esophageal variceal bleeding.

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### INTRODUCTION

Variceal bleeding is a catastrophic complication of liver cirrhosis. Although the short-term mortality of variceal bleeding has improved due to recent advances in treatment, the long-term outcomes remain guarded.

Currently, endoscopic treatment remains the predominant method for prevention and treatment of variceal bleeding. Endoscopic treatments include band ligation and injection sclerotherapy. Injection sclerotherapy with *N*-butyl-2-cyanoacrylate (Histoacryl, B-Braun Surgical GmbH, Melsungen, Germany) has been successfully used for variceal bleeding, but Histoacryl injection is associated with a variety of complications, some of which can be disastrous<sup>[1]</sup>.



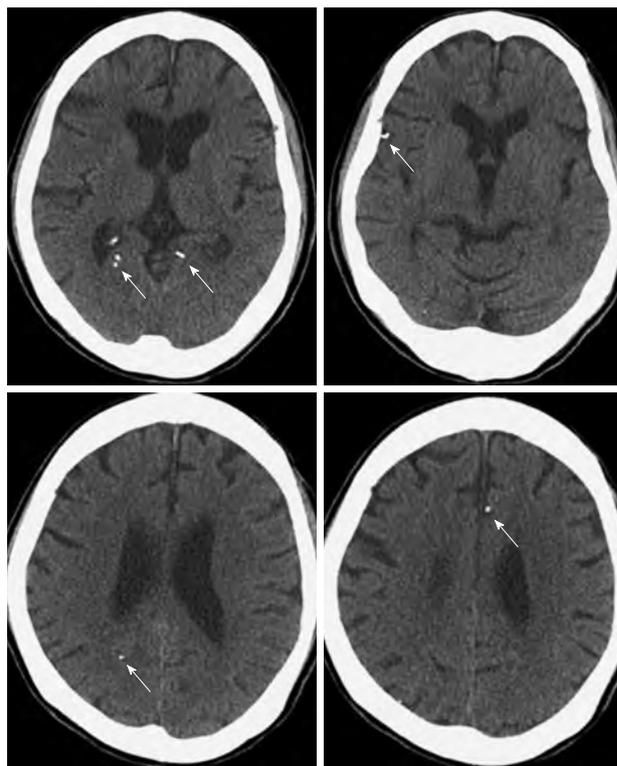
**Figure 1** Endoscopy showing a large esophageal varix. The varix occupies more than half of the esophageal lumen. The adherent, whitish fibrin plug on top of the varix is considered a site of recent hemorrhage.

Systemic embolization including the cerebrum, lung, spleen, and portal vein is a rare and serious complication of Histoacryl injection that has been principally described in the treatment of gastric variceal bleeding<sup>[2,3]</sup>. However, this complication following the esophageal variceal injection of Histoacryl is extremely rare. To date, few cases of this complication at one site have been reported in the English literature.

To our knowledge, this is the first report of a case of multiple embolizations including the cerebrum and spleen after Histoacryl injection in esophageal variceal bleeding.

## CASE REPORT

A 55-year-old woman with alcohol-induced liver cirrhosis of Child-Pugh class B was admitted to Chonnam National University Hwasun Hospital (Jeonnam, South Korea) with a 1-d history of hematemesis. She denied prior gastrointestinal bleeding, peptic ulcer diseases, and use of ulcerogenic medications. On admission, she had a pulse of 90 beats/min, a blood pressure of 80/50 mmHg, and a respiratory rate of 30 breaths/min. The head and neck examination was normal, except for anemic conjunctiva. She had florid spider angiomas. The abdomen was non-tender with ascites, and the spleen tip was slightly palpable. Rectal examination demonstrated the presence of maroon-colored, liquid stool. Laboratory studies revealed the following: hemoglobin, 8.2 g/dL (normal range, 12-18 g/dL); hematocrit, 24.8% (37%-52%); white blood cell count, 9300/mm<sup>3</sup> (4000-10800/mm<sup>3</sup>); platelet count, 100000/mm<sup>3</sup> (130000-450000/mm<sup>3</sup>); total protein, 5.1 g/dL (5.8-8.1 g/dL); albumin, 2.3 g/dL (3.1-5.2 g/dL); total bilirubin, 1.4 mg/dL (0.3-1.3 mg/dL); aspartate aminotransferase, 90 U/L (7-38 U/L); and alanine aminotransferase, 8 U/L (6-42 U/L). Her coagulation profiles were prothrombin time 19.6 s (11-14.9 s) and activated partial thromboplastin time 34.5 s (28-40 s). Endoscopy showed a large esophageal varix with a fibrin plug (Figure 1). Because the bleeding esophageal varix was too large to apply band ligation, we performed injection sclerotherapy



**Figure 2** Non-contrast brain computed tomography showing multiple high attenuation lesions (arrows). The multiple high attenuation lesions are emboli of the Histoacryl-Lipiodol mixture. The high attenuation lesions were seen in the frontal lobe and the parieto-occipital lobe.

with a mixture of Histoacryl and Lipiodol (Laboratoire Guerbet, Aulnay-Sous-Bois, France). The mixture was injected intra-variceally using a 21-gauge needle injector (Injector Force, NM-200L-0821, Olympus Optical Co., Ltd., Tokyo, Japan). The mixture consisted of 0.5 mL of Histoacryl and 0.5 mL of Lipiodol. Because variceal bleeding was not controlled after the first injection, a second injection was performed in the same manner. After the second injection, variceal bleeding was controlled. The total injected volume was 2 mL. However, she developed dysarthria and right motor weakness (grade III/V) 1 h after the injection. Brain computed tomography (CT) showed multiple hyperdense foci in the frontal lobe and the parieto-occipital lobe (Figure 2). Magnetic resonance imaging of the brain showed acute multifocal cortical infarctions. Abdominal CT revealed several wedge-shaped, low attenuation lesions in the spleen, indicating infarction (Figure 3). To evaluate the cause of the newly developed cerebral and splenic infarctions, a transcranial Doppler (TCD) bubble test was performed. The TCD bubble test is used to detect a right-to-left shunt. We used 2 MHz M-mode TCD (ST3, Spencer Technologies, Seattle, Washington, United States; SONARA, Viasys Healthcare, Conshohocken, Pennsylvania, United States) to detect microbubbles in the middle cerebral artery. TCD demonstrated the presence of a microbubble on the M-mode displays in the middle cerebral artery. TCD using Spencer Logarithmic Scale Grades was indicative of grade III during resting and grade IV during the Valsalva maneuver

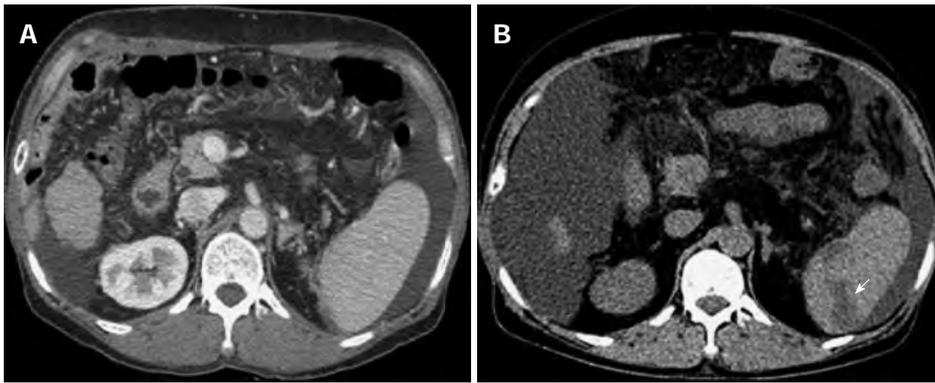


Figure 3 Abdominal computed tomography reveals several wedge-shaped, low attenuation lesions in the spleen, indicating infarction (arrow). A: Computed tomography (CT) before endoscopic treatment; B: CT after endoscopic treatment.

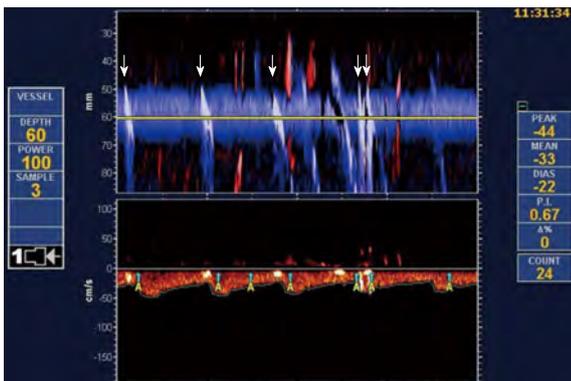


Figure 4 Transcranial Doppler bubble test showing the Doppler flow through the middle cerebral artery. The embolus is clearly represented in the power M-mode (upper panel) as a whitish sloping track. The whitish sloping track means that microemboli disrupt the ultrasonic signal (arrows).

(Figure 4). In the Spencer Logarithmic Scale Grades, grade I and II are considered negative for patent foramen ovale, and grades III through V are considered positive. These findings indicate that the patient had a patent foramen ovale. Therefore, cerebral and splenic infarctions may develop due to emboli cause by a right-to-left shunt. At the follow-up examination, her neurologic symptoms were improved, but neurologic sequelae remained.

## DISCUSSION

Endoscopic treatments, such as band ligation and injection sclerotherapy, became the cornerstone of the management of variceal bleeding. Endoscopic band ligation is the preferred form of endoscopic treatment for esophageal variceal bleeding, but its application in actively bleeding patients is still challenging because the bands at the endoscope tip limit the operator's field of vision. Injection sclerotherapy with various sclerosants is recommended in patients in whom endoscopic band ligation is not technically feasible. Several sclerosants are available, including 5% sodium morrhuate, 1%-3% sodium tetradecyl sulfate, 5% ethanolamine oleate, and 0.5%-1% polidocanol<sup>[2]</sup>. Adhesives such as Histoacryl have been used successfully for the treatment of variceal bleeding.

Injection sclerotherapy provides effective treatment for variceal bleeding, but it has been associated with a

variety of complications. Minor complications including chest pain, dysphagia, fever, and esophageal ulcer are common, although not typically serious. Uncommon and serious complications include bacteremia, esophageal perforation, mediastinitis, and brain abscess. Rarely, systemic embolic complications have followed injection sclerotherapy, and these can be disastrous<sup>[4]</sup>. Systemic embolic complications following Histoacryl injection have been reported, and the common sites of embolic complications were the lung, spleen, cerebrum, and portal vein. Additionally, this complication has been principally described in the treatment of gastric variceal bleeding. Because most gastric varices are associated with a gastrorenal and splenorenal shunts<sup>[5]</sup>, blood flow is abundant, and Histoacryl injection is likely to cause systemic embolization due to the migration of the agent through a shunt<sup>[6]</sup>. In contrast to gastric variceal injection, systemic embolic complications arising from esophageal variceal injection sclerotherapy are extremely rare; to date, only three cases of cerebral embolic complications following esophageal variceal injection sclerotherapy have been documented in the literature<sup>[7,8]</sup>.

Because the bleeding esophageal varix was too large to apply band ligation, we performed injection sclerotherapy with Histoacryl. Cerebral and splenic infarctions followed the bleeding esophageal variceal injection sclerotherapy. Ours is an additional case of cerebral infarction caused by the esophageal variceal injection of Histoacryl, although it is the first report of a case of multiple embolizations including the cerebrum and spleen after the esophageal variceal injection of Histoacryl. The possible explanation for the development of systemic emboli may be the transient patent foramen ovale caused by the episodes of coughing, which induced a temporary right-to-left shunt.

Clearly, transesophageal echocardiography (TEE) is considered the gold standard for right-to-left shunt diagnosis, but it is poorly tolerated by patients and sometimes requires sedation. Additionally, TEE limits the patient's ability to perform a Valsalva maneuver<sup>[9]</sup>. Because our case had a large esophageal varix, we performed a TCD bubble test rather than TEE. The TCD bubble test has proven to be a trustworthy and less invasive method for diagnosing a right-to-left shunt<sup>[10]</sup>. In our case, the ultrasound waves were reflected by microbubbles on the TCD bubble

test, indicating the patent foramen ovale. Additionally, the TCD bubble test was grade III during resting and grade IV during the Valsalva maneuver, according to the Spencer Logarithmic Scale<sup>[8]</sup>. Therefore, the cerebral and splenic infarctions in our case may have been caused by emboli via the patent foramen ovale.

Factors that increase embolization risk include the size of varices, the presence of a collateral vessel, excessive dilution, rapid polymerization, large volume (> 1 mL/injection) and rapid Histoacryl injection. Our case had the three possible embolic risk factors including the large size of the varices, the large volume (> 1 mL) of the mixture injected, and rapid injection<sup>[6]</sup>. Because most embolization risks associated with Histoacryl, as described above, are preventable, proper injection technique may help minimize the risk of serious complications and improve the long-term outcome.

Taken together, although injection sclerotherapy with Histoacryl is a relatively safe and efficacious procedure for the treatment of variceal bleeding, serious complications such as systemic embolization can occur. Ours is the first report of a case of multiple embolizations including the cerebrum and spleen after Histoacryl injection to treat esophageal variceal bleeding. Systemic embolization, despite its rarity, should be considered among the serious complications of Histoacryl injection for the treatment of esophageal variceal bleeding.

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**S- Editor** Wen LL **L- Editor** A **E- Editor** Li JY



## Acute iatrogenic Budd-Chiari syndrome following hepatectomy for hepatolithiasis: A report of two cases

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Author contributions: Liang TB designed the study; Bai XL, Chen YW, Zhang Q and Ye LY conducted the majority of study and wrote the manuscript; Xu YL, Wang L, Cao CH, Gao SL, Khodoruth MAS and Ramjaun BZ revised the manuscript; Dong AQ offered suggestions for this work.

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sidered a rare complication following hepatectomy for hepatolithiasis. Awareness of potential hepatic outflow obstructions and timely management are critical to avoid poor outcomes when performing hepatectomy for hepatolithiasis.

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**Key words:** Acute iatrogenic Budd-Chiari syndrome; Hepatolithiasis; Hepatectomy; Inferior vena cava

**Core tip:** The occurrence of acute iatrogenic Budd-Chiari syndrome (BCS) following hepatectomy for hepatolithiasis is rarely reported. However, it may occur following a particularly difficult hepatectomy for complicated hepatolithiasis. Here, we report two cases of acute BCS and present our clinical experience in managing these cases.

Bai XL, Chen YW, Zhang Q, Ye LY, Xu YL, Wang L, Cao CH, Gao SL, Khodoruth MAS, Ramjaun BZ, Dong AQ, Liang TB. Acute iatrogenic Budd-Chiari syndrome following hepatectomy for hepatolithiasis: A report of two cases. *World J Gastroenterol* 2013; 19(34): 5763-5768 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i34/5763.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i34.5763>

### Abstract

Budd-Chiari syndrome (BCS) is defined as hepatic venous outflow obstruction at any level from the small hepatic veins to the junction of the inferior vena cava (IVC) and the right atrium, regardless of the cause of obstruction. We present two cases of acute iatrogenic BCS and our clinical management of these cases. The first case was a 43-year-old woman who developed acute BCS following the implantation of an IVC stent for the correction of stenosis in the IVC after hepatectomy for hepatolithiasis. The second case was a 61-year-old woman with complete obstruction of the outflow of hepatic veins during bilateral hepatectomy for hepatolithiasis. Acute iatrogenic BCS should be con-

### INTRODUCTION

Budd-Chiari syndrome (BCS) is characterized by hepatic venous outflow obstruction at the level of the hepatic venules, the large hepatic veins (HV), the inferior vena cava (IVC), or the right atrium<sup>[1]</sup>. Obstruction of the hepatic venous outflow tract, which leads to hepatic congestion as blood flows into, but not out of the liver, results in damage to the hepatic parenchymal cells and the cells lining the hepatic sinusoids. BCS can cause liver dysfunction and even lead to liver failure.

There are multiple known causes of BCS, including both heritable and acquired hypercoagulable states, compression or invasion of the IVC, as well as other less common miscellaneous causes<sup>[2-4]</sup>. BCS can also be idiopathic. Here, we report two cases of acute BCS with an uncommon cause and present our clinical experience in managing these cases.

## CASE REPORT

### Case 1

A 43-year-old woman developed acute BCS following the implantation of an IVC stent for correction of stenosis in the IVC after hepatectomy for hepatolithiasis. The patient was admitted with recurrent cholangitis. Magnetic resonance cholangiopancreatography showed lithiasis in the intra- and extra-hepatic bile duct, dilatation of the biliary tract and enlargement of the gallbladder. In addition, computed tomography (CT) (Figure 1) demonstrated right lobe, caudate lobe and segment II (Counauid's classification) atrophy of the liver. After biliary decompression by endoscopic retrograde cholangio-pancreatography and control of infection, the following procedures were performed: right hemi-hepatectomy, segment II resection, caudate lobectomy, cholecystectomy, choledochotomy, and choledochojejunostomy. During right hepatectomy, the IVC was accidentally wedge resected due to the obscure boundary between the IVC and the right lobe of the liver, which was immediately sutured.

After surgery, the patient developed congestive hepatopathy, manifested as rapidly progressive abdominal distention, and severe pitting edema of the lower back and lower limbs. Magnetic resonance venography 3 d after operation (Figure 2) showed obvious stenosis near the entrance of the three HVs into the IVC, at the same location where the wedge was resected and repaired. Emergent balloon dilation was performed using a balloon catheter and a metallic stent (30 mm × 75 mm), guided by digital subtraction angiography (Figure 3). However, after this procedure, the patient's liver function deteriorated (Table 1). Contrast enhanced CT confirmed hepatic congestion and revealed that the proximal end of the stent was directly at the entrance of the HV into the IVC. Doppler ultrasonography showed reduced blood flow velocity in the HVs, the maximum velocity in the middle HV was 14 cm/s. There was turbulence in the left HV and backflow in the portal vein. Based on these findings, the patient was diagnosed with acute BCS, caused by the improper position of the stent which blocked the entrance of the HV into the IVC.

Following this diagnosis, the patient was immediately scheduled for surgery to remove the metallic stent. The stenosis of the IVC was 5 cm long and the diameter of the lumen was only 1 cm. The stenosis was repaired using a BalMedic (Beijing Balance Medical Co. Ltd, China) pericardial patch (2 cm × 5 cm) under cardiopulmonary bypass and deep hypothermic circulatory arrest (Figure 4). After surgery, the clinical manifestations of BCS

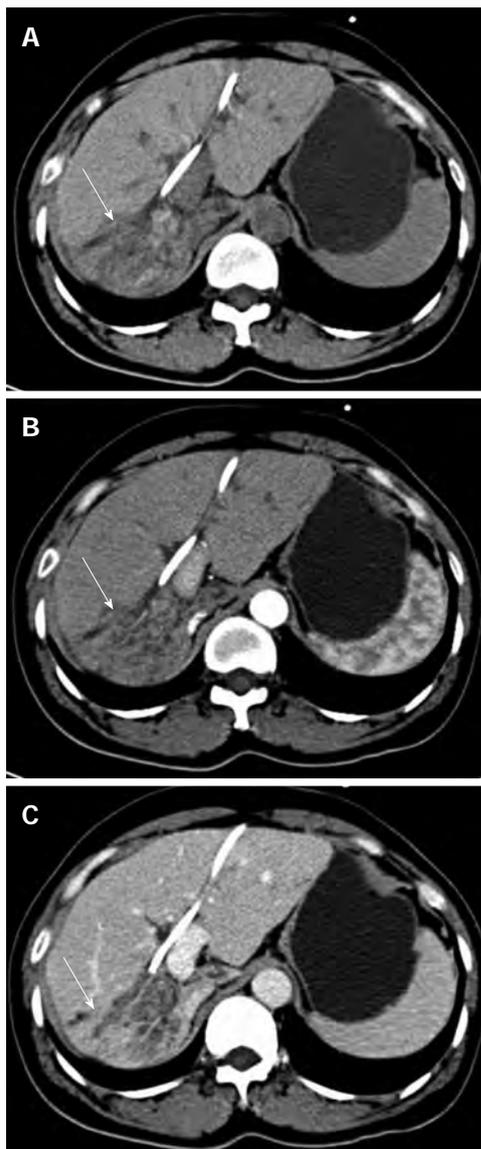


Figure 1 Preoperative computed tomography scan shows hepatolithiasis in the right liver with hepatic parenchymal atrophy (arrow). A: Non-contrast; B: Arterial phase; C: Venous phase.

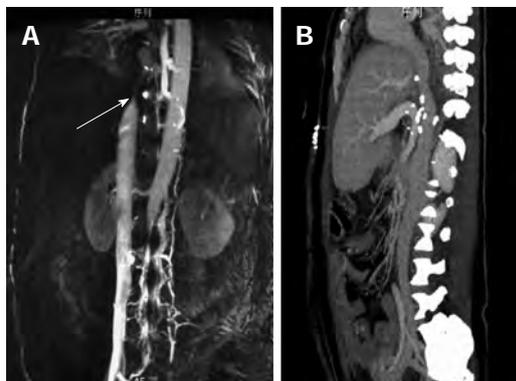


Figure 2 The inferior vena cava. A: Pretreatment of stenosis in inferior vena cava (IVC) on magnetic resonance venogram imaging (arrow); B: One year after patch repair, computed tomography angiography showed no obvious obstruction in the IVC and good hepatic vein outflow.



Figure 3 Treating stenosis of the inferior vena cava by balloon dilation and stent placement. A: Digital subtraction angiography shows the stenosis of inferior vena cava; B: Balloon dilation; C: After balloon angioplasty; D: Metallic stenting.

Table 1 Temporal liver function data for case 1				
	Before hepatectomy	After hepatectomy	After metallic stent placement	5 d after IVC patch repair
TBIL (μmol/L)	26.7	45.2	83.3	26.3
DBIL (μmol/L)	13.6	34.3	45.6	17.3
IBIL (μmol/L)	13.1	15.8	37.7	9
ALT (U/L)	146	294	335	24
AST (U/L)	73	199	287	16
WBC (10 <sup>6</sup> /L)	4.0	10.3	5.9	6.0
Hb (g/L)	107	81	80	88
PLT (10 <sup>6</sup> /L)	117	51	57	73
PT (s)	11	18.4	20.5	16.0
INR	0.92	1.53	1.7	1.33

TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; WBC: White blood cell; Hb: Hemoglobina; PLT: Platelet; PT: Prothrombin time; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

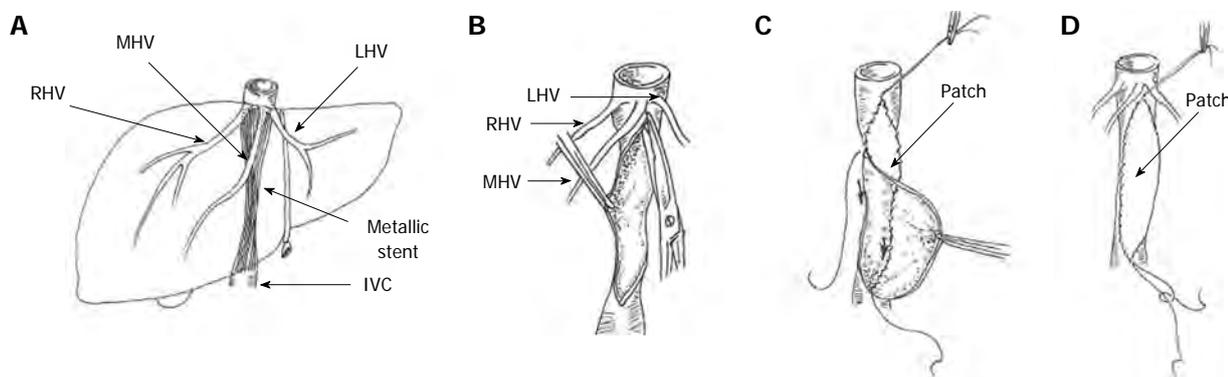
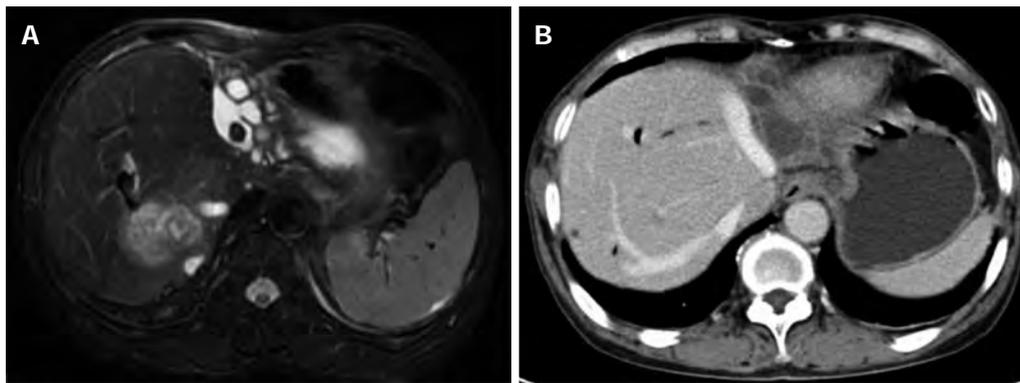


Figure 4 Diagram of the operative procedure for removing the metallic stent from the inferior vena cava and repairing the stenosis with a BalMedic pericardial patch. A: The proximal end of the stent was directly at the entrance of the hepatic veins into the inferior vena cava (IVC); B: Open the stenosis of IVC; C: BalMedic pericardial patch was anastomosed to the IVC to broaden its lumen; D: After patch repair of the IVC. LHV: Left hepatic vein; MHV: Middle hepatic vein; RHV: Right hepatic vein.

were alleviated, liver function improved, and the patient recovered without further complications (Table 1). Anticoagulation therapy with low molecular weight heparin (LMWH) for 3 d followed by Warfarin for 3 mo was administered. At one year follow-up, the patient had no recurrent cholangitis, no symptoms related to BCS, and normal liver function. CT angiography showed no obvious stenosis in the IVC (Figure 2).

**Case 2**

A 61-year-old woman experienced complete obstruction of the outflow of HVs during bilateral hepatectomy for hepatolithiasis. She underwent an open cholecystectomy and choledocholithotomy with T-tube drainage 15 years previously for cholelithiasis. In recent years, she suffered from recurrent cholangitis. Imaging findings (Figure 5) showed bilateral hepatolithiasis, dilation of the biliary



**Figure 5 Preoperative imaging findings.** A: Magnetic resonance imaging shows bilateral hepatolithiasis with infection at the posterior right lobe of the liver; B: Major hepatic vein on computed tomography.

tract, common bile duct stones, and a hypodense area on CT in the posterior lobe of the right liver, suggesting infection.

During surgery, the liver was found to be distorted with marked atrophy of the right posterior hepatic lobe and left half of the liver. In addition, the right anterior hepatic lobe (segment V and VIII), and the right caudate lobe showed significant hyperplasia. No neoplasms were found in the wall of the bile duct during choledochoscopy. The liver was then freed to begin resection of the left part of the liver, the right posterior lobe and the right caudate lobe. The left half was resected first. Then, the right HV was revealed and divided using a 45-mm endoscopic linear stapler (Echelon 45 ENDOPATH Stapler, Ethicon ENDO-Surgery, Cincinnati, Ohio, United States). Immediately after ligation, there was sudden and significant congestion of the liver, and hepatic outflow blockage was suspected. The liver was examined and stenosis was found in the middle HV, which was thought to be the left HV. Thus, we set out to repair the middle HV and the right HV.

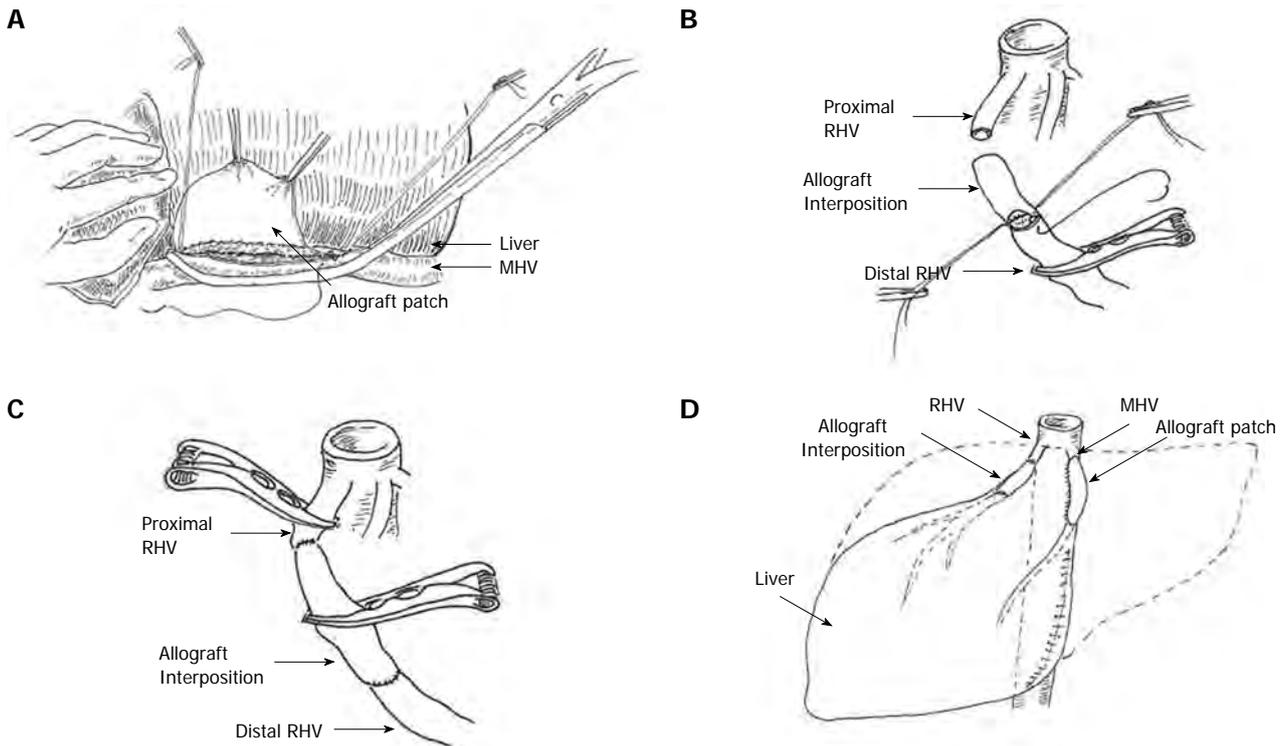
The stenosed middle HV was opened and cryopreserved (-80 °C). A common iliac artery allogeneic graft about 3 cm was prepared. The lumen of the artery was opened and anastomosed to the MHV forming a patch to broaden the stenosed middle HV. Liver swelling was observed to subside after middle HV reconstruction. Both ends of the right HV were then clamped using vascular clamps to control bleeding, and a 1-2 cm allogeneic iliac vein graft was used to repair the right HV (Figure 6). The outflow of blood from the liver was then assessed; no thrombosis or air embolism was found and the liver swelling subsided. It should be noted that the hepatic blood inflow of the porta hepatis was blocked several times during this procedure. The longest blockage time was 40 min with a total length of 60 min. During the procedure, the liver was cold conditioned using ice to attenuate warm ischemic injury. A T-tube was placed in the common bile duct for drainage and to improve accessibility for the future removal of residual stones in the biliary tract. Due to the episode of HV obstruction, a right posterior lobectomy was not performed.

Postoperatively, peak liver enzymes aspartate aminotransferase 380 U/L and alanine aminotransferase 407 U/L were detected, with normal total bilirubin at postoperative day 2. Liver enzymes decreased to the normal range within one week. Postoperative recovery was uneventful. Anticoagulation therapy with LMWH for 3 d followed by Warfarin for 3 mo was administered. Postoperative CT scan (Figure 7) showed no obstruction or thrombosis in the right or middle HV.

## DISCUSSION

BCS is a rare clinical condition characterized by hepatic venous outflow obstruction due to various causes<sup>[1]</sup>. The most common causes of BCS are hypercoagulable states, other uncommon causes have been identified such as tumor invasion, otherwise its origins are idiopathic<sup>[5]</sup>. There are also sporadic reports of BCS as a surgical complication after liver transplantation<sup>[5]</sup>, and right hepatectomy resulting from torsion of the remnant liver<sup>[6]</sup>. In this study, we report two cases of acute BCS following hepatectomy for hepatolithiasis.

Hepatolithiasis is a common disease in Asian countries<sup>[7]</sup>. A multimodal approach has been advocated in the management of this condition, including endoscopic, percutaneous, or open surgery. Hepatectomy is considered the most effective treatment and is indicated in some cases, especially those with recurrent bacterial cholangitis and irreversible atrophy of parts of the liver<sup>[8-10]</sup>. Despite the efficacy of this approach, higher morbidity and mortality is associated with hepatectomy for hepatolithiasis<sup>[9]</sup>. As observed in the two reported cases, performing a partial hepatectomy for hepatolithiasis is a particularly difficult and challenging procedure because of the dense perihepatic adhesions (due to recurrent cholangitis or previous surgery), and abnormal intrahepatic anatomy (due to the atrophy-hypertrophy complex)<sup>[11]</sup>. These challenges require detailed preoperative evaluation to avoid venous injury, including high resolution imaging to identify the intrahepatic anatomy, as well as the relationship between the liver and the IVC. In addition, due to co-existing atrophy and regeneration of the liver, the func-



**Figure 6** Diagram of reconstruction of middle and right hepatic vein. A: Middle hepatic vein (MHV) reconstruction using a common iliac artery allogenic graft; B: Right hepatic vein (RHV) reconstruction using an allogeneic iliac vein graft; C: Completion of RHV reconstruction using allograft interposition; D: Accomplishment of MHV and RHV outflow.



**Figure 7** Postoperative computed tomography scan. A, B: Demonstrated good right hepatic vein tract; C: The inter-positioned graft using an allogeneic iliac vein (arrow).

tion of the HV is frequently abnormal, thus it is crucial to ligate the major HV for testability before continuing with the procedure. Based on our experience with hepatic outflow obstruction, we believe that awareness of possible complications and timely management are critical for attenuating liver congestion and salvaging the liver.

The treatment of BCS is based on its cause, consisting of anticoagulation therapy, thrombolytic therapy, radiological procedures (*i.e.*, angioplasty, stenting or transjugular intrahepatic portosystemic shunts), surgical decompression, surgical shunts, and surgical correction of the lesion. Rarely, liver transplantation may be necessary if the liver is irreparably damaged<sup>[12,13]</sup>. Of note, balloon angioplasty and stent placement has been increasingly favored and the effectiveness of these procedures has been documented<sup>[1,14]</sup>. However, in the first case presented

above, the stenosis was very close to the secondary porta of the liver, and the placement of the stent was incorrect as it blocked the entrance of the HV into the IVC. Thus, based on our experience, surgical broadening of the stricture of the IVC is strongly advocated. With regard to our second case, it is evident that no other method is a substitute for surgical reconstruction of the HV to resolve hepatic congestion.

In conclusion, episodes of acute iatrogenic BCS following hepatectomy for hepatolithiasis are a rare occurrence. Awareness of potential hepatic outflow obstructions and timely management are critical to avoid poor outcomes when performing hepatectomy for hepatolithiasis. From our experience, prompt surgical reconstruction of the HV should be favored to salvage the congested liver.

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## Immunoglobulin G4-related gastrointestinal diseases, are they immunoglobulin G4-related diseases?

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### Abstract

In immunoglobulin G4 (IgG4)-related disease (RD), organ enlargement or nodular lesions consisting of abundant infiltration of lymphocytes and IgG4-positive plasma cells and fibrosis are seen in various organs. Although infiltration of many IgG4-positive plasma cells is detected in the gastric and colonic mucosa and major duodenal papilla of patients with autoimmune pancreatitis, it cannot be diagnosed as a gastrointestinal lesion involved in IgG4-RD, because none of the following is observed in these lesions: a mass-like formation; dense fibrosis; or obliterative phlebitis. Based on our review of the literature, there appear to be two types of IgG4-

related gastrointestinal disease. One is a gastrointestinal lesion showing marked thickening of the wall of the esophagus and stomach, consisting of dense fibrosis with abundant infiltration of IgG4-positive plasma cells, which usually show submucosal spreading. The other is an IgG4-related pseudotumor occurring in gastrointestinal regions such as the stomach, colon, and major duodenal papilla, showing polypoid or mass-like lesions. Most solitary IgG4-related gastrointestinal lesions that are not associated with other IgG4-RD appear to be difficult to diagnose. It is of utmost importance to rule out malignancy. However, these lesions may respond to steroid therapy. To avoid unnecessary resection, IgG4-related gastrointestinal diseases should be considered in the differential diagnosis.

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**Key words:** Immunoglobulin G4; Autoimmune pancreatitis; Gastritis; Colonic polyp; Ulcerative colitis

**Core tip:** Although the concept of immunoglobulin G4 (IgG4)-related gastrointestinal disease remains unclear, there appear to be two types of IgG4-related gastrointestinal disease. One is a gastrointestinal lesion showing marked thickening of the wall of the esophagus and stomach, consisting of dense fibrosis with abundant infiltration of IgG4-positive plasma cells, which usually show submucosal spreading. The other is an IgG4-related pseudotumor occurring in gastrointestinal regions such as the stomach, colon, and major duodenal papilla, showing polypoid or mass-like lesions. It is of utmost importance to rule out malignancy. To avoid unnecessary resection, IgG4-related gastrointestinal diseases should be considered in the differential diagnosis.

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## INTRODUCTION

Immunoglobulin G4 (IgG4)-related disease (RD) is a recently recognized systemic condition characterized by elevated serum IgG4 levels and steroid responsiveness. IgG4-RD shows organ enlargement or nodular lesions consisting of abundant infiltration of lymphocytes and IgG4-positive plasma cells and fibrosis. IgG4-RD affects various organs such as the pancreas, bile duct, gallbladder, liver, salivary gland, lacrimal gland, retroperitoneum, and lymph nodes simultaneously or metachronously. IgG4-RD frequently presents both clinically and radiologically with findings that mimic a malignancy, resulting in unnecessary resection<sup>[1-4]</sup>. According to comprehensive clinical diagnostic criteria for IgG4-RD<sup>[4]</sup>, IgG4-RD is diagnosed when there is a characteristic diffuse/localized swelling or mass in a single or multiple organs with elevated serum IgG4 levels, or there are histological findings of abundant infiltration of IgG4-positive plasma cells and lymphocytes, along with fibrosis.

Autoimmune pancreatitis (AIP) is a typical lesion of IgG4-RD, and the concept of IgG4-RD was proposed based on research on AIP<sup>[1,2]</sup>. Although it has been reported that infiltration of many IgG4-positive plasma cells was observed in the gastric mucosa, colonic mucosa, and major duodenal papillae of some AIP patients<sup>[5-10]</sup>, it is questionable whether they are the lesions involved in IgG4-RD. To clarify IgG4-related gastrointestinal disease, this article reviews the published literature about the relationships between IgG4 and gastrointestinal diseases such as esophagitis, gastritis, colitis, and duodenal papillitis with abundant infiltration of IgG4-positive plasma cells. A PubMed database search, from 1990 to April, 2013, using the terms “autoimmune pancreatitis or IgG4-related” and “esophagus, duodenum, papilla, colon” identified 116 papers. Additional sources were identified by scanning the bibliographies of original and review articles.

## IGG4-RELATED ESOPHAGEAL LESIONS

There have been two case reports of IgG4-related esophagitis<sup>[11,12]</sup>. In both cases, esophageal stricture with thickening of the esophageal wall evoked debilitating dysphagia and weight loss. Endoscopy showed esophageal stricture without a cancerous lesion. With a diagnosis of gastrointestinal stromal tumor based on endoscopic ultrasound-guided fine needle aspiration (one case) and because of concerns regarding a hidden malignancy (one case), esophageal resection was performed in both patients. On gross examination, the resected specimens showed an esophageal submucosal stricture with mucosal

ulceration and wall thickening; histologically, they showed transmural chronic fibrotic inflammation with abundant infiltration of IgG4-positive plasma cells and lymphocytes and phlebitis. There was no evidence of other IgG4-RD. The post-operative serum IgG4 level was 138 mg/dL in one case. Both lesions are considered esophageal manifestations of IgG4-RD and should be called IgG4-related esophagitis. These lesions would probably respond to steroid therapy. Thus, IgG4-related esophagitis should be kept in mind in the differential diagnosis of unexplained esophagitis with stricture.

## IGG4-RELATED GASTRIC LESIONS

It has been reported that infiltration of many IgG4-positive plasma cells was observed in the gastric mucosa in 33%-47% of AIP patients<sup>[10,13]</sup>. Shinji *et al*<sup>[14]</sup> and Uehara *et al*<sup>[15]</sup> also reported that IgG4-positive plasma cells were significantly more abundant in the gastric mucosa of AIP patients. Most of the infiltrated IgG4-positive plasma cells in the gastric mucosa disappeared in the biopsy specimen from the gastric mucosa after steroid therapy<sup>[16]</sup>. However, neither dense fibrosis nor obliterative phlebitis was observed in the gastric mucosa of AIP patients. Baez *et al*<sup>[17]</sup> reported a patient with AIP and IgG4-related sialadenitis who showed diffusely thickened (up to 1.4 cm) and nodular gastric mucosa with abundant infiltration of IgG4-positive plasma cells. The patient's serum IgG4 level was within the normal range (58 mg/dL), but the gastric lesion improved after steroid therapy. Kaji *et al*<sup>[18]</sup> reported an AIP patient (IgG4 level, 595 mg/dL) with multiple sporadic polyps in the gastric body with erosion and redness on the surface containing many infiltrated IgG4-positive plasma cells. On the other hand, two 3-cm-sized submucosal tumors that were laparoscopically wedge-resected showed histological findings of storiform fibrosis with abundant infiltration of lymphocytes and IgG4-positive cells (> 50/hpf), and they were reported as IgG4-related inflammatory pseudotumor of the stomach<sup>[19]</sup>. Both cases showed normal serum IgG4 levels and no evidence of other IgG4-RD<sup>[19]</sup>. Rollins *et al*<sup>[20]</sup> also reported a laparoscopically resected 5.6-cm IgG4-related fibrosclerosing pseudotumor of the stomach. Three cases with well-circumscribed, sclerosing nodular lesions of the stomach composed of fibrous tissue with abundant infiltration of IgG4-positive plasma cells were reported, and they were not associated with other IgG4-RD<sup>[21,22]</sup>. Fujita *et al*<sup>[23]</sup> reported a case with refractory gastric ulcers that worsened after successful *Helicobacter pylori* eradication therapy. The biopsy specimens taken from the ulcers showed abundant infiltration of IgG4-positive plasma cells (50/hpf). The patient's serum IgG4 level was elevated to 203 mg/dL, but he had no other IgG4-RD.

Bateman *et al*<sup>[24]</sup> reported a case of intractable gastric ulcer showing storiform fibrosis and abundant infiltration of IgG4-positive plasma cells (> 100/hpf). These reported lesions are considered IgG4-related gastric lesions. Anjiki *et al*<sup>[25]</sup> reported that gastric emptying assessed by

the carbon 13 acetate breath test was impaired in AIP patients and improved to the reference range after steroid therapy, and they suggested that the stomach might be a target organ of IgG4-RD.

## IGG4-RELATED MAJOR DUODENAL PAPILLARY LESIONS

It has been reported that the duodenal major papilla is swollen in 41%-65% of AIP patients<sup>[26-28]</sup>. Abundant infiltration of IgG4-positive plasma cells is reportedly detected in 55%-80% of AIP patients<sup>[8,10,26,27]</sup>. Both a swollen major papilla and abundant infiltration of IgG4-positive plasma cells have shown improvement after steroid therapy<sup>[8,29]</sup>. In the resected pancreas of AIP patients, lymphoplasmacytic inflammation with many IgG4-positive plasma cells was detected in the major duodenal papilla connected to the head of the pancreas; thus, IgG4 immunostaining of biopsy specimens obtained from the major duodenal papilla might be useful to support the diagnosis of AIP<sup>[8,26,27,30,31]</sup>. Hisa *et al.*<sup>[32]</sup> reported a resected case of a lymphoplasmacytic granuloma with abundant IgG4-positive plasma cells localized to the major duodenal papilla. The case was not associated with other IgG4-RD. This lesion is considered to be an IgG4-related pseudotumor localized to the major duodenal papilla.

## IGG4-RELATED COLONIC LESIONS

Although infiltration of many IgG4-positive plasma cells is occasionally detected in the colonic mucosa of AIP patients, dense fibrosis or obliterative phlebitis was not observed in the lesion<sup>[1,5-7,21,33]</sup>. Although Ravi *et al.*<sup>[34]</sup> suggested that inflammatory bowel disease might represent an extrapancreatic manifestation of AIP, in general, conventional AIP (type 1 AI) is rarely associated with ulcerative colitis (UC)<sup>[2,35]</sup>. IgG4-positive plasma cell infiltration is sometimes detected in the colonic mucosa of UC patients<sup>[36-40]</sup>, but the mechanisms underlying IgG4-positive plasma cell infiltration in the colonic mucosa of UC patients are unknown. Matsui *et al.*<sup>[41]</sup> reported a case of an AIP patient with a colonic polyp (ascending colon) containing many IgG4-positive plasma cells<sup>[42]</sup> who developed colonic polyposis (descending colon) containing many IgG4-positive plasma cells 1 year after complete remission of AIP with steroid therapy. The polyposis was markedly reduced with re-administration of steroids. They suggested that enhanced T helper type 2 responses to intestinal microflora may underlie the immunopathogenesis in patients with IgG4-RD<sup>[43]</sup>. Well-circumscribed sclerosing nodular lesions of the cecum and sigmoid colon composed of hyalinized fibrocollagenous tissue with abundant infiltration of IgG4-positive plasma cells were reported, and the two cases had no other IgG4-RD<sup>[21]</sup>. These polypoid or nodular lesions appear to be IgG4-related colonic lesions.

## IGG4-RELATED INFLAMMATORY PSEUDOTUMOR OF AN ILEAL CONDUIT

An ill-defined, fibrotic, tumor-like mass, histologically showing fibrosis with infiltration of lymphocytes and IgG4-positive plasma cells and marked obliterative phlebitis, occurred in an ileal conduit created as part of surgery for urinary bladder cancer<sup>[44]</sup>.

## DISCUSSION

IgG4-RD shows organ enlargement or nodular lesions consisting of abundant infiltration of lymphocytes and IgG4-positive plasma cells and fibrosis in various organs simultaneously or metachronously<sup>[3,4]</sup>. The first International Symposium on IgG4-RD held in 2011 suggested that the term "IgG4-related disease" aptly recognizes the ubiquity of IgG4 within involved organs, and proposes a style that employs "IgG4-related" as a prefix to the organ system affected and pathological guidelines for the diagnosis of IgG4-RD<sup>[3,45]</sup>. The diagnosis of IgG4-RD rests on the combined presence of the characteristic histopathological appearances and increased number of IgG4-positive plasma cells. A histologically high suspicion of IgG4-RD requires the presence of at least two of three characteristic histological features including (1) dense lymphoplasmacytic infiltration; (2) fibrosis, usually storiform in character; and (3) obliterative phlebitis. The IgG4 counts required for the diagnosis differ among affected organs, ranging from 10 to 200 cells/hpf. The diagnosis of IgG4-RD requires considering both histopathological findings and clinical information such as elevated serum IgG4 levels, other organ involvement that is consistent with IgG4-RD, and effective response to steroid therapy<sup>[45]</sup>.

Comprehensive clinical diagnostic criteria for IgG4-RD<sup>[4]</sup> were proposed in 2011. In the criteria, IgG4-RD is diagnosed when there is a characteristic diffuse/localized swelling or mass in a single or multiple organs with elevation of serum IgG4 levels or IgG4-related histological findings. However, the concept of IgG4-related gastrointestinal diseases was not included as objects of the criteria. It is unclear whether IgG4-related gastrointestinal diseases exist or what gastrointestinal lesions are regarded as IgG4-RD. To clarify these questions, this review of IgG4-related gastrointestinal diseases, the first of its kind, was conducted.

Infiltration of many IgG4-positive plasma cells is detected in the gastric and colonic mucosa and the major duodenal papillae of some AIP patients, but none of the following are observed in these lesions: a mass-like formation; dense fibrosis; or obliterative phlebitis<sup>[5-10]</sup>. They cannot be diagnosed as gastrointestinal lesions involved in IgG4-RD, because, as in many other organ systems, increased IgG4-positive plasma cells do not mean the disease is one of the family members of IgG4-RD. At this point, both the clinical finding of mass forming and histological finding of abundant infiltration of IgG4-

positive plasma cells with fibrosis would appear to be necessary to make the diagnosis of IgG4-related gastrointestinal diseases.

IgG4-related pseudotumors have been reported in several organs, such as the liver and lung<sup>[46-48]</sup>. On review of these papers, there appear to be two types of IgG4-related gastrointestinal disease. One is a gastrointestinal lesion showing marked thickening of the wall of the esophagus<sup>[11,12]</sup> and stomach<sup>[17,23,24]</sup>, consisting of dense fibrosis with abundant infiltration of IgG4-positive plasma cells, which usually show submucosal spreading. The other is an IgG4-related pseudotumor occurring in gastrointestinal regions such as the stomach<sup>[18-22]</sup>, colon<sup>[21,42]</sup>, and major duodenal papilla<sup>[52]</sup>, showing polypoid or mass-like lesions. We currently consider these lesions to be IgG4-related gastrointestinal diseases. However, this is the first review of a few cases of IgG4-related gastrointestinal diseases; further studies should be conducted to confirm this concept.

Most solitary IgG4-related gastrointestinal lesions that are not associated with other IgG4-RD appear to be difficult to diagnose. It is of utmost importance to rule out malignancy. However, these lesions may respond to steroid therapy. To avoid unnecessary resection, IgG4-related gastrointestinal diseases should be considered in the differential diagnosis.

## CONCLUSION

The concept of IgG4-related gastrointestinal disease remains unclear due to its rarity. There appear to be some IgG4-related gastrointestinal lesions that present with a mass-like lesion consisting of abundant infiltration of IgG4-positive plasma cells and lymphocytes and fibrosis.

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## Pathophysiology, epidemiology, classification and treatment options for polycystic liver diseases

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### Abstract

Polycystic liver diseases (PLD) represent a group of genetic disorders in which cysts occur in the liver (autosomal dominant polycystic liver disease) or in combination with cysts in the kidneys (autosomal dominant polycystic kidney disease). Regardless of the genetic mutations, the natural history of these disorders is alike. The natural history of PLD is characterized by a continuous increase in the volume and the number of cysts. Both genders are affected; however, women have a higher prevalence. Most patients with PLD are asymptomatic and can be managed conservatively. Severe symptoms can affect 20% of patients who develop massive hepatomegaly with compression of the surrounding organs. Rarely, patients with PLD suffer from acute

complications caused by the torsion of hepatic cysts, intraluminal cystic hemorrhage and infections. The most common methods for the diagnosis of PLD are cross sectional imaging studies. Abdominal ultrasound and computerized tomography are the two most frequently used investigations. Magnetic resonance imaging is more sensitive and specific, and it is a valuable test for patients with intravenous contrast allergies or renal dysfunction. Different treatment modalities are available to physicians caring for these patients. Medical treatment has been ineffective. Percutaneous sclerotherapy, transarterial embolization, cyst fenestration, hepatic resection and liver transplantation are indicated to specific groups of patients and have to be tailored according to the extent of disease. This review outlines the current knowledge of the pathophysiology, clinical course, diagnosis and treatment strategies of PLD.

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**Key words:** Polycystic liver disease; Hepatic; Epidemiology; Classification; Therapy; Genetic

**Core tip:** The management of patients with symptomatic polycystic liver disease is challenging. Among several treatments options, the most common interventions are: percutaneous cyst aspiration, fenestration, hepatic resection and liver transplantation. There is no consensus on the best treatment options and the optimal timing for interventions in symptomatic patients. In vision of these limitations, we reviewed the most recent literature and present a comprehensive article on this topic.

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## INTRODUCTION

The association between polycystic liver disease (PLD) and autosomal dominant polycystic kidney disease (ADPKD) was described for the first time by Bristowe in 1856<sup>[1,2]</sup>. Initially, it was thought that PLD could develop only in the context of ADPKD<sup>[3]</sup>. The notion that isolated PLD might be a separate condition was proposed in the 1950s<sup>[4]</sup>. In 2003, a linkage analysis of eight Finnish families confirmed that PLD is genetically distinct from ADPKD<sup>[5]</sup>. Asymptomatic patients usually do not require any intervention<sup>[6]</sup>. In some patients, massive hepatomegaly can cause pain or compression of the adjacent gastrointestinal organs, vasculature, and diaphragm. This can have a significant effect on patients' quality of life and performance status<sup>[6,7]</sup>. For these patients, the main aim is to reduce their symptoms by decreasing the liver volume<sup>[8-10]</sup>. Current surgical options include open or laparoscopic cyst fenestration with or without hepatic resection and orthotopic liver transplantation (OLT). Significant advances in surgical techniques have improved the outcomes of PLD patients. However, the selection of the appropriate approach remains a clinical challenge, and there is no consensus on the optimal timing and what represents the best therapeutic modality.

## INCIDENCE AND GENETICS

ADPKD affects up to 0.2% of the general population<sup>[11]</sup>. On the other hand, isolated PLD has prevalence of less than 0.01%<sup>[12]</sup>. Both ADPKD and PLD are autosomal dominant and 75%-90% of patients with ADPKD have associated PLD<sup>[13]</sup>. In humans, PLD has been linked to mutations of four genes. Two genes (*PKD1*, locus 16p13.3, encoding polycystin-1 and *PKD2*, locus 4q21, and encoding polycystin-2) are predominantly associated with renal disease and less frequently with PLD. *PKD1* mutations are more common and account for 85%-90% of the cases, whereas mutations in *PKD2* affect approximately 10%-15% of patients<sup>[11]</sup>. The remaining two mutations (*PRKCSH*, locus 19p13.2, encoding the protein kinase C substrate 80K-H or hepatocystin and *SEC63*, locus 6q21, encoding the Sec63 protein) are linked only to the development of PLD<sup>[11]</sup>. However, these mutations explain just 25% to 40% of cases of PLD<sup>[14,15]</sup>. Comparative characteristics between ADPKD and PLD are summarized in Table 1.

## PATHOPHYSIOLOGY

Malformation of the hepatic ductal plate and cilia of cholangiocytes is the main characteristic linked to the pathophysiology of PLD (Figure 1).

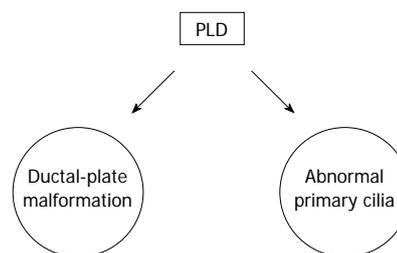
### DUCTAL-PLATE MALFORMATION

The ductal plate is the anatomical template for the development of the intra-hepatic bile ducts<sup>[16]</sup>. Normal

**Table 1 Comparative epidemiological and genetic mutation characteristics of autosomal dominant polycystic kidney disease associated polycystic liver disease and isolated polycystic liver disease**

Characteristics	ADPKD associated PLD	Isolated PLD
Prevalence	0.20%	< 0.01%
Type of inheritance	AD	AD
Gene mutated	<i>PKD1</i> ; <i>PKD2</i>	<i>PRKCSH</i> ; <i>SEC63</i>
Encoded product	Polycystin-1; Polycystin-2	Hepatocystin; Sec63 protein
Chromosome locus	216p13.3; 4q21	19p13.2; 6q21

AD: Autosomal dominant; ADPKD: Autosomal dominant polycystic kidney disease; PLD: Polycystic liver disease.



**Figure 1 Pathophysiology of polycystic liver disease.** PLD: Polycystic liver disease.

bile ducts arise from the ductal plate through a complex sequence of growth and apoptosis. Complexes of disconnected intralobular bile ductules (von Meyenburg complexes) are retained because they do not undergo apoptosis in PLD<sup>[10]</sup>. As a consequence, multiple cysts arise from progressive dilatation of these abnormal ductules<sup>[17-19]</sup> that display the same epithelium and structures of functioning cholangiocytes<sup>[20,21]</sup>.

### ABNORMAL PRIMARY CILIA

Cholangiocytes are the only ciliated cells in the liver. Cilia have mechanosensory capacity and modulate the intracellular levels of cAMP and Ca<sup>2+</sup> when bent by the flow of bile. They also detect changes in osmolarity and composition of the bile<sup>[22-24]</sup>. Ciliary defects result in a decreased cytoplasmic level of Ca<sup>2+</sup> and an increased cytoplasmic level of cAMP<sup>[25]</sup>. These changes are responsible for the hyperproliferation of cholangiocytes and for the cystogenesis that is a consequence of the altered balance between fluid secretion and absorption in the lumen of the biliary ducts<sup>[25]</sup>.

## NATURAL HISTORY AND RISK FACTORS FOR PLD

The natural history of PLD is characterized by a continuous increase in the volume and the number of cysts<sup>[11,26,27]</sup>. The annual growth of affected livers is in the range of 0.9%-3.2% of the initial hepatic volume<sup>[10,28-30]</sup>. Both genders are affected; however, women have a higher

**Table 2 Risk factors for liver-cyst growth in polycystic liver disease**

Risk factors for liver-cyst growth in polycystic liver disease
Advancing patient age
Female gender
Estrogen exposure: multiple pregnancies, OCPs, estrogen replacement therapy
Severity of renal dysfunction and renal cyst volume

OCPs: Oral contraceptive pills.

prevalence. Exposure to estrogen during pregnancies, the use of oral contraceptive pills or estrogen replacement therapy seems to accelerate the progression of the disease<sup>[1,27,31]</sup>. Other risk factors are the severity of renal dysfunction that is dependent on the volume of the cysts in the kidneys<sup>[1]</sup>. Table 2 summarizes the known factors that influence the progression of PLD.

### CLINICAL PRESENTATION

PLD is asymptomatic in 80% of patients<sup>[8,9]</sup> and is usually diagnosed incidentally. Women present with massive and symptomatic cystic liver more frequently than men<sup>[32]</sup>. For 20% of patient, symptoms are typically caused by the compression of organs surrounding the liver, bleeding or infectious complications of the cysts. Compressive symptoms include abdominal distention, early satiety that can lead to decreased oral intake and severe malnutrition, gastro-esophageal reflux, dyspnea, hepatic venous-outflow obstruction (Budd-Chiari syndrome), inferior vena cava syndrome, portal-vein and bile-duct compression. Complications of liver cysts include infections, torsions, rupture and hemorrhage<sup>[1,18,33,34]</sup> (Table 3). In asymptomatic patients, serum laboratory studies are usually normal. In the presence of symptoms, 47% of patients have elevated serum alkaline phosphatase, 70% have elevated serum levels of gamma glutamil transferase<sup>[35-38]</sup>, 27% have elevated serum levels of aspartate amino transferase and 15% have elevated serum levels of total bilirubin<sup>[35,36]</sup>. Liver synthetic function is typically preserved despite the presence of innumerable cysts<sup>[52]</sup> while 45% of patients might have elevated serum tumor marker CA19-9 without proof of malignancy<sup>[39]</sup>. Other tumor markers such as CA-125, carcinoembryonic antigen, and alpha-fetoprotein may also be elevated but less frequently than CA19-9<sup>[40-42]</sup>.

### ASSOCIATED EXTRA-HEPATIC DISEASES

Intracranial arterial aneurysms can affect 6% of patients without a family history of ADPKD and up to 16% of patients with family history of ADPKD. Other common conditions are mitral-valve prolapse and colonic diverticulosis that can be detected in 25% of patients with PLD<sup>[1,11,43-45]</sup>. Screening for intracranial aneurysm by

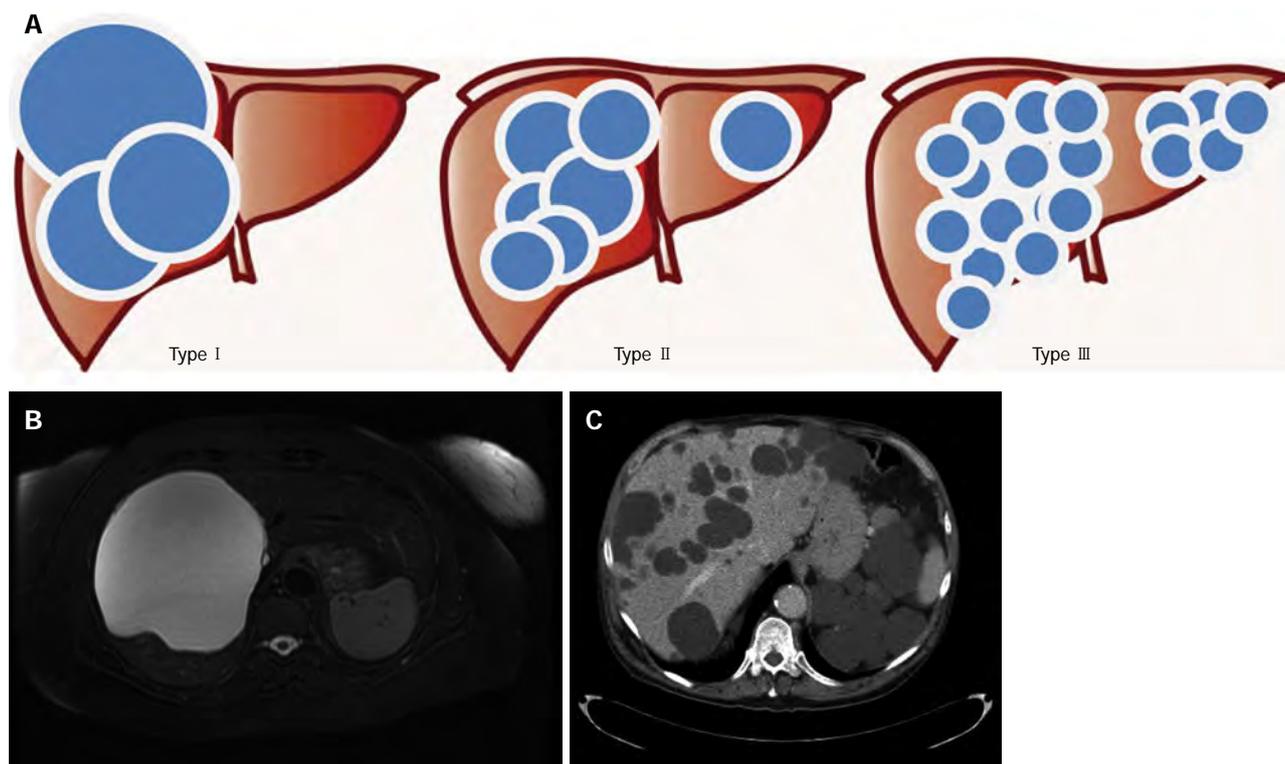
**Table 3 Summary of the most frequent symptoms caused by polycystic liver disease**

Symptoms due to mass effect	Symptoms due to complications of the cysts
Abdominal distention	Infection
Early satiety	Torsion
Postprandial fullness	Rupture
Gastro-oesophageal reflux	Haemorrhage
Malnutrition	
Dyspnoea	
Hepatic venous-outflow obstruction (Budd-Chiari syndrome)	
Inferior vena cava syndrome	
Portal-vein compression	
Bile-duct compression	

magnetic resonance angiography (MRA) is recommended only for patients with ADPKD, older than 30 years or for those patients with family history of hemorrhagic strokes or intracranial arterial aneurysms<sup>[46]</sup>. Screening for intracranial arterial aneurysms is also warranted in cases of a sudden severe headache, or for candidates to liver or kidney transplantation. Screening for mitral-valve prolapse is not recommended unless a cardiac murmur is ascultated during routine clinical examinations<sup>[11,47]</sup>. Finally, patients with ADPKD may have asymptomatic cysts within other organs, such as the pancreas, spleen, ovaries, and lungs<sup>[48]</sup>. Pancreatic cysts are the most common with a reported incidence of 9% among ADPKD patients older than 30 years<sup>[49-51]</sup>.

### DIAGNOSIS

The most common methods for the diagnosis of PLD are cross sectional imaging studies. Abdominal ultrasound (US) and computerized tomography (CT) are the two most frequent investigations<sup>[52,53]</sup>. For hepatic cysts, MRI is more sensitive and specific, and it is a valuable test for patients with intravenous contrast allergies or renal dysfunction or when other studies are unable to satisfy the diagnostic needs<sup>[54]</sup>. Hepatic cysts have radiological characteristics identical to benign developmental cysts. On US, they appear anechoic and well-circumscribed<sup>[55]</sup>. On CT and MRI, they have non-enhancing, well-circumscribed round walls with hypodense content<sup>[55]</sup>. On T2-weighted MRI and CT scans, they appear homogenously enhanced spherical lesions<sup>[55]</sup> (Figure 2B and C). The distinction between isolated PLD and ADPKD relies on the number of renal cysts, age at presentation and family history (Table 4). In adults, younger than 30 years with a positive family history, the diagnosis of ADPKD is established by radiologic evidence of at least two unilateral or bilateral cysts. At least two cysts in each kidney are necessary for the diagnosis of patients between the age of 30 to 59 years, and at least four cysts in each kidney for patients 60 years or older<sup>[56]</sup>. It is worth noting that at least one third of patients with isolated PLD may also have a few kidney cysts<sup>[15,56,57]</sup>. It has been proposed that



**Figure 2** Gigot's classification for polycystic liver diseases. A: Graphical representation; B: Abdominal magnetic resonance imaging of a patient affected by Gigot I cystic liver disease; C: Abdominal computerized tomography of a patient affected by Gigot II cystic liver disease.

**Table 4** The ravine diagnostic criteria for autosomal dominant polycystic kidney disease

Patient's age (yr)	Number of cysts	
	Positive family history	Negative family history
≤ 30	At least 2 cysts affecting 1 or both kidneys	At least 5 cysts
31-59	At least 2 cysts in each kidney	At least 5 cysts
≥ 60	At least 4 cysts in each kidney	At least 8 cysts

sporadic cases of PLD should be diagnosed when a patient has more than 15 to 20 cysts and no previous family history<sup>[1,18]</sup> while four cysts suffice in the presence of a positive familial history<sup>[1,18]</sup>.

### INFECTED CYSTS

Hepatic cysts may become infected, and cause life-threatening sepsis<sup>[58,59]</sup>. Often, infected hepatic cysts are responsible for recurrent episodes of fever without any other signs or symptoms. In these circumstances, the diagnosis can be quite difficult as the accuracy of imaging tests remain low due to the altered anatomy of the liver parenchyma<sup>[60]</sup>. A promising investigation technique for suspected infected hepatic cysts is In-111 WBC scan<sup>[61]</sup>. Several other tracers such as 99mTc-diphosphonates, 67Gacitrate, and 111In- or 99mTc-labeled leukocytes have also been used<sup>[62]</sup>. Although labeled leukocyte imaging is theoretically the test of choice for detecting most infections, it is labor intensive, not always available and

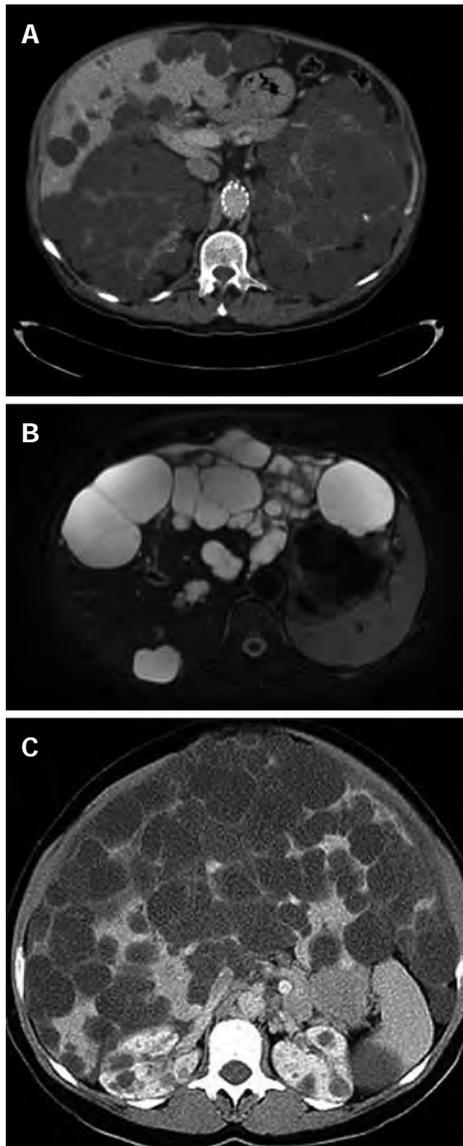
involves direct handling of potentially infected blood products. Therefore, considerable effort has been devoted to search for alternatives to this procedure such as the use of 67Gallium scintigraphy and 18F-FDG-positron emission tomography (PET). In recent years, PET has become the most commonly used diagnostic test for the detection of infected renal and hepatic cysts<sup>[60,62,63]</sup>. However, the accuracy of this technique is still under investigation. The literature on the treatment of infected cysts in PLD patients is very scarce and based only on a few case reports. Most of patients will need parenteral broad spectrum antibiotic therapy with percutaneous drainage of the content of the cyst if their symptoms persists.

### CLASSIFICATION

Several clinical classifications have been proposed to grade the severity of PLD.

### GIGOT'S CLASSIFICATION

Gigot's classification relies on imaging findings and was designed to identify the best candidates for fenestration of symptomatic cysts<sup>[38]</sup> (Figure 3): Type I : presence of less than 10 large hepatic cysts measuring more than 10 cm in maximum diameter. Type II : diffuse involvement of liver parenchyma by multiple cysts with remaining large areas of non-cystic liver parenchyma. Type III: presence of diffuse involvement of liver parenchyma by small and medium-sized liver cysts with only a few areas of



**Figure 3** Gigot's classification relies on imaging findings and was designed to identify the best candidates for fenestration of symptomatic cysts. A: Intravenous contrast enhanced computerized tomography (CT) of a patient affected by polycystic liver and renal disease. The cysts appear hypodense with smooth and regular walls; B: T2 magnetic resonance imaging of a patient with multiple hepatic cysts. The cystic fluid appears bright on T2 images; C: Abdominal CT of a patient affected by Gigot III cystic liver disease.

normal liver parenchyma.

### QUIAN'S CLASSIFICATION

Qian's classification has been used in the context of familial screening and relies on the number of cysts and the presence of symptomatic hepatomegaly<sup>[18]</sup>: (1) grade 0 - 0 cysts; (2) grade 1 - 1 to 10 cysts; (3) grade 2 - 11 to 20 cysts; (4) grade 3 - more than 20 cysts; and (5) grade 4 - more than 20 cysts and symptomatic hepatomegaly.

### SCHNELLDORFER'S CLASSIFICATION

Schnelldorfer's classification aims at differentiating pa-

tients who could benefit from resection or transplantation as summarized in Table 5<sup>[64]</sup>.

### TREATMENT

Most patients with PLD are asymptomatic and do not require any intervention<sup>[6]</sup>. However, symptomatic PLD patients might require treatment when they experience severe dysfunction of organs around the liver due to the increased hepatic volume or when one or more cysts get tormented, infected or develop intra-cystic hemorrhages (Table 6).

### AVOIDANCE OF EXPOSURE TO ESTROGENS

Observational and experimental studies have shown that PLD may worsen under the influence of estrogen during pregnancy or when patients are prescribed estrogen replacement therapy<sup>[1,27,31]</sup>. Estrogen can increase both the number of liver cysts and their volume, therefore, hormonal therapy should be stopped in most symptomatic patients when appropriate<sup>[27]</sup>.

### NON-SURGICAL TREATMENTS

Medical management may be valuable in symptomatic patients with Gigot's type II/III.

### SOMATOSTATIN ANALOGUES

Somatostatin analogues are inhibitors of cAMP and they reduce the secretion of fluid and the proliferation of many cell types, including cholangiocytes<sup>[65-69]</sup>. They also suppress the expression of insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and other cytogenetic growth factors<sup>[70]</sup>. In addition, somatostatin analogues inhibit the downstream signaling of these receptors<sup>[70]</sup>. Two randomized controlled trials have recently demonstrated that after 6 to 12 mo, treatment with lanreotide, a long-acting somatostatin analogue, was associated with a significant reduction of liver volume in patients with PLD compared with placebo<sup>[28,29]</sup>. However, the average hepatic volume reduction was only 3% to 5%. The severity of abdominal symptoms was also not significantly improved<sup>[28]</sup>. Currently, somatostatin analogues are indicated only for a selected group of patients with symptomatic PLD in whom the risks for surgical intervention are not justified, or in whom the surgical intervention is technically challenging.

### MAMMALIAN TARGET OF RAPAMYCIN INHIBITORS

Mammalian target of rapamycin (m-TOR) inhibitors have immunosuppressive and antiproliferative effects<sup>[71]</sup>. Sirolimus and Everolimus were studied in Phase-II prospective randomised control trials. None of the two drugs showed substantial therapeutic effects both in hu-

**Table 5 Summary of Schnelldorfer’s classification that aims at differentiating patients who could benefit from resection or transplantation**

	Type A	Type B	Type C	Type D
Symptoms	Absent or mild	Moderate or severe	Severe (or moderate)	Severe (or moderate)
Cyst characteristics	Any	Limited No. large cysts	Any	Any
Areas of relative normal liver parenchyma	Any	≥ 2 sectors	≥ 1 sector	< 1 sector
Presence of portal vein or hepatic vein occlusion in the preserved hepatic sectors	Any	Absent	Absent	Present
Recommended therapy	Observation or medical therapy	Cyst fenestration	Partial hepatectomy with possible fenestration of remnant cysts	Liver Transplantation

**Table 6 Summary of treatment options for polycystic liver disease**

Treatment approach	Treatment type
Nonsurgical	Medical
	Somatostatin analogues
	mTOR inhibitors
	Interventional radiology:
Surgical	Arterial embolization
	Percutaneous sclerotherapy
	Fenestration
	Hepatic resection with fenestration
	Liver transplantation

OCP’s: Oral contraceptive pills; mTOR: Mammalian target of rapamycin.

mans<sup>[72-74]</sup> and in animal models<sup>[75]</sup>. Clinical prospective data on the effect of m-TOR inhibitors are currently not available, and this class of medications should not be recommended outside clinical trials.

## INTERVENTIONAL RADIOLOGY: ARTERIAL EMBOLIZATION

Trans-catheter arterial embolization has been used since the early 2000s<sup>[76]</sup>. Hepatic artery branches supplying the hepatic segments replaced by the cysts are targeted by using microcoils or polyvinyl alcohol particles measuring 150-250 μm in diameter<sup>[76,77]</sup>. For patients with advanced PLD and multilobar disease, trans-catheter arterial embolization can be technically demanding. The largest series of patients treated with this modality included 30 patients who had a significant reduction of the volume of their cysts ( $6.667 \pm 2.978 \text{ cm}^3$  down to  $4.625 \pm 2.299 \text{ cm}^3$ ), whereas the volume of the unaffected hepatic parenchyma increased<sup>[76]</sup>. After several months, patients reported improvement of their symptoms and no major complications except for occasional post-embolization syndrome<sup>[76,77]</sup>.

## PERCUTANEOUS SCLEROTHERAPY

This technique requires radiologically guided percutaneous aspiration of the content of the cysts followed by the injection of a sclerosing agent that inhibit the reaccumulation of fluid by damaging the epithelial lin-

ing the cysts<sup>[78,79]</sup>. Symptomatic patients with one to five large dominant cysts (Gigot’s type I) are suitable for percutaneous sclerotherapy. Most commonly, cysts with a diameter larger than 5 cm are candidates for this treatment<sup>[10]</sup>. Puncturing of the cyst can be done with a 5 or 7 French catheter<sup>[80]</sup> and sclerosing agents commonly used include ethanol, ethanolamine oleate, minocycline and tetracycline. Although a single session is often sufficient, some patients require more than one<sup>[81]</sup>. Aspiration with sclerotherapy has an excellent safety profile, although severe abdominal pain can be caused by peritoneal irritation due to spillage of the sclerosing agent<sup>[10]</sup>. The majority of patients who undergo percutaneous sclerotherapy has improved symptoms in the immediate period following the procedure<sup>[10]</sup>, but only 20% will have partial, or full regression of their disease<sup>[10]</sup>.

## SURGERY

Patient and treatment selection remain a clinical challenge. There is no consensus on selection criteria for surgery, the optimal timing, and technique. Current surgical options include fenestration, partial liver resection and OLT. Fenestration and partial liver resection are options for Gigot’s type I and II patients. For Gigot’s type III disease, fenestration and partial liver resection are often ineffective, and OLT should be considered as it is the only curative treatment. In general, several factors have to be considered before any surgical intervention is recommended: (1) The degree of cystic burden; (2) The distribution of the cysts; and (3) The proximity of the cysts to the main biliary ducts and portal and hepatic vein branches.

## SURGICAL PEARLS

In Gigot’s type I or II, symptoms might not be related to the size of the entire liver but to the size of one or two large cysts. These patients can be treated similarly to those with simple cysts. Some hepatic segments such as V and VI are frequently spared and, therefore, surgical resection can be performed if the spared liver parenchyma is thought to be sufficient. Frequently, the right hepatic veins are compressed by cysts causing the formation of collateral circulation between the right and the middle hepatic veins that can be responsible for intraoperative bleeding during the parenchymal transaction.

**Table 7** Summary of largest series published on the surgical techniques used for cystic fenestration of symptomatic polycystic liver disease

Ref.	No. of patients	Technique	Outcome	Complications	Follow-up (mo)
van Erpecum <i>et al</i> <sup>[35]</sup>	15	Open fenestration	0% symptom recurrence	One mortality	Mean of 48
Kabbej <i>et al</i> <sup>[37]</sup>	13	Lap fenestration	72% symptom recurrence	54% morbidity	Mean follow-up 26
Gigot <i>et al</i> <sup>[38]</sup>	10	Open fenestration	11% symptom recurrence	60% morbidity	73 mean follow-up
van Keimpema <i>et al</i> <sup>[82]</sup>	12	Lap fenestration	Reduction in liver volume by 12.5%	Bile leak, vena cava occlusion and sepsis	-
Pirenne <i>et al</i> <sup>[92]</sup>	4	Lap fenestration	100% symptom relief	50% cyst recurrence	-
Liska <i>et al</i> <sup>[95]</sup>	7	Lap fenestration plus open	-	No mortality	Mean 41
Bai <i>et al</i> <sup>[96]</sup>	10	Lap fenestration	Symptom and cyst recurrence in 20%	3 patients with minor complications. No mortality	Mean of 57
Palanivelu <i>et al</i> <sup>[97]</sup>	4	Lap fenestration	100% cyst recurrence	-	-
Garcea <i>et al</i> <sup>[98]</sup>	6	Lap/Open fenestration	16.7% symptom recurrence, 33.3% cyst recurrence	50% morbidity	5-36
Neri <i>et al</i> <sup>[99]</sup>	3	Lap fenestration	100% symptom relief	50% morbidity	-
Kornprat <i>et al</i> <sup>[100]</sup>	8	Lap fenestration	0% symptom recurrence	-	-
Robinson <i>et al</i> <sup>[101]</sup>	11	Lap fenestration	54.5% symptom recurrence	-	-
Fiamingo <i>et al</i> <sup>[102]</sup>	6	Lap fenestration	30% symptom recurrence	50% morbidity	1-64
Tocchi <i>et al</i> <sup>[103]</sup>	18	Lap/open fenestration	-	-	-
Kopera <i>et al</i> <sup>[104]</sup>	39	Open fenestration (n = 34); Lap (n = 5)	21% symptom recurrence	-	75 mean follow-up
Morino <i>et al</i> <sup>[105]</sup>	7	Lap fenestration	40% symptom recurrence	44% morbidity rate	-
Farges <i>et al</i> <sup>[106]</sup>	13	Open fenestration	23% symptom recurrence	69% morbidity	84 follow-up
Ueno <i>et al</i> <sup>[118]</sup>	13	Open fenestration (n = 6); Lap (n = 13)	71% symptom recurrence	30% morbidity	37 mean follow-up

Lap: Laparoscopic.

## FENESTRATION

Fenestration is a surgical technique that combines aspiration and surgical unroofing of the cyst. It has the advantage that multiple cysts can be treated in one session<sup>[48,82]</sup>. Fenestration is effective in symptomatic patients with Gigot's type I and II disease<sup>[83]</sup>. Patients with superficial and a limited number of large cysts are the best candidates for this procedure<sup>[48]</sup>. Fenestration may be achieved by laparotomy or laparoscopy<sup>[48]</sup>. Patients with the majority of their cysts located in the right posterior segments (VI, VII), or at the dome of the liver (segment VIII) may be better candidates for open fenestration because these cysts are difficult to be visualized and fenestrated by laparoscopic approach<sup>[48]</sup>. Published series describing open and laparoscopic fenestration are summarized in Table 7. Immediate symptom relief is achieved in 92% of the patients, whereas up to 25% experience recurrence of the cysts or symptoms<sup>[10]</sup>. Complication rate after fenestration is in the range of 23% while mortality is about 2%<sup>[10]</sup>. Complications include ascites, pleural effusion, hemorrhage and bile leakage<sup>[84]</sup>. Factors that predict failure of fenestration are previous abdominal procedures, deep-seated cysts, incomplete unroofing, cysts in segments VII-VIII, and the presence of diffuse PLD<sup>[10]</sup>.

## HEPATIC RESECTION WITH FENESTRATION

Hepatic resection is usually reserved for highly symptomatic patients who are incapacitated by their disease due to the massive expansion of their livers (Gigot's type II and

III)<sup>[38]</sup>. In these circumstances fenestration alone is rarely successful because the liver parenchyma is rigid and it does not collapse<sup>[10]</sup>. Symptom relief is achieved in 86% of cases although cyst recurrence is expected in one third of patients<sup>[10]</sup>. Overall, most of the patients have an improvement in their quality of life and functional status<sup>[36]</sup>. The morbidity rate associated with this procedure can be up to 50% and includes ascites, pleural effusions, biliary leakage, and hemorrhage<sup>[10]</sup>. One of the reasons for these complications is the fact that there is a significant distorsion of the intra-hepatic vasculature and biliary tree which makes these procedures technically very challenging. Mortality rate is around 3%<sup>[10]</sup>. As subsequent adhesions may complicate future OLT, this surgical treatment is usually preserved for patients with massive hepatomegaly for which OLT is not an option<sup>[85,86]</sup>. Published series describing hepatic resection with/without fenestration for symptomatic PLD are summarized in Table 8.

## LIVER TRANSPLANTATION

OLT is the only curative treatment for patients with severe PLD<sup>[87]</sup>. It is indicated in those patients with disabling symptoms that lead to decreased performance status and quality of life<sup>[10]</sup>. Patients with PLD usually have normal liver function and the current organ allocation system based on the Model for End-Stage Liver Disease (MELD) is often unable to assist this group of patients. For these patients, MELD exception criteria are needed<sup>[88,89]</sup>. Because of the shortness of available grafts, the need for life-long immunosuppression and the perioperative risks, OLT is indicated only for symptomatic patients

**Table 8 Summary of largest series published on the surgical techniques used for cystic fenestration and resection of symptomatic polycystic liver disease**

Ref.	No.	Technique	Outcome	Complications	Follow-up (mo)
Que <i>et al</i> <sup>[36]</sup>	31	Fenestration and resection	3% symptom recurrence	3% mortality, 58% morbidity	Mean of 28
Schnelldorfer <i>et al</i> <sup>[64]</sup>	124	Fenestration and resection	93% symptom relief, 72.6% recurrent cyst formation	72.6% morbidity, 3.2% mortality	Mean of 48
Kornprat <i>et al</i> <sup>[100]</sup>	9	Fenestration and resection	100% symptom relief, 11% recurrence	33.35% morbidity	24-98
Kopera <i>et al</i> <sup>[104]</sup>	5	Fenestration and resection	0% symptom recurrence	-	-
Li <i>et al</i> <sup>[107]</sup>	21	Fenestration and resection	14.3% cyst recurrence	76.2% cyst morbidity, 0% mortality	10-155
Gamblin <i>et al</i> <sup>[108]</sup>	51	Fenestration and resection	3.9% symptom recurrence	17.6% morbidity, no mortality	1-49
Yang <i>et al</i> <sup>[109]</sup>	7	Fenestration and resection	100% symptom recurrence	100% morbidity, no mortality	Mean of 20
Vons <i>et al</i> <sup>[110]</sup>	12	Resection	17% symptom recurrence	8% mortality, 83% morbidity	Mean of 34
Soravia <i>et al</i> <sup>[111]</sup>	10	Fenestration and resection	33% symptom recurrence	10% mortality, 20% morbidity	Mean of 69
Henne-Bruns <i>et al</i> <sup>[112]</sup>	8	Fenestration and resection	50% symptom recurrence	No mortality, 38% morbidity	Mean of 15
Vauthey <i>et al</i> <sup>[113]</sup>	5	Fenestration and resection	0% symptom recurrence	0% mortality, 100% morbidity	Mean of 14
Sanchez <i>et al</i> <sup>[114]</sup>	9	Resection	100% symptom relief, 100% recurrence	0% mortality	Mean of 35
Newman <i>et al</i> <sup>[115]</sup>	9	Fenestration and resection	88.9% symptom relief, 0% recurrence	11.1% mortality, 55.6% morbidity	2-44
Iwatsuki <i>et al</i> <sup>[116]</sup>	9	Resection	44.4% symptom relief, 44.4% recurrence	0% mortality, 33.3% morbidity	12-180

**Table 9 Summary of largest series published on the outcomes of patients undergoing liver transplantation for symptomatic polycystic liver disease**

Ref.	No. of patients	Previous surgery	Combined liver and kidney transplantation	Morbidity	Mortality	Follow-up (mo)	Re-transplantation
Pirenne <i>et al</i> <sup>[92]</sup>	16	25%	6%	38%	13%	Range 18-120	0%
Taner <i>et al</i> <sup>[117]</sup>	13	-	54%	85%	31%	-	0%
Ueno <i>et al</i> <sup>[118]</sup>	14	-	36%	64%	21%	-	0%
Ueda <i>et al</i> <sup>[119]</sup>	3	-	0	33%	0%	Mean of 32	0%
Gustafsson <i>et al</i> <sup>[120]</sup>	7	57%	43%	57%	43%	Mean of 4	0%
Swenson <i>et al</i> <sup>[121]</sup>	9	44%	33%	44%	11%	Mean of 26	11%
Lang <i>et al</i> <sup>[122]</sup>	17	35%	47%	47%	29%	Mean of 12	12%
Washburn <i>et al</i> <sup>[123]</sup>	5	90%	20%	0%	20%	Mean of 38	0%
Starzl <i>et al</i> <sup>[124]</sup>	4	0%	25%	0%	50%	Mean of 38	0%

**Table 10 Suggested management strategies based on Gigot's classification**

Gigot's I	Gigot's II - III
Percutaneous sclerotherapy	Hepatic resection with fenestration if feasible
Fenestration	Liver transplantation

with Gigot's type II and III disease<sup>[12,48,90]</sup>. For patients undergoing OLT for PLD, perioperative morbidity is 40%-50%, whereas overall mortality is 10%-17%<sup>[10]</sup>. In 3% of patients, retransplantation is required<sup>[10]</sup> and combined renal and liver transplantation are necessary in 42% of patients<sup>[91,92]</sup>. Expected survival at 1- and 5-year are 93% and 92% for patients undergoing OLT alone while for patients who undergo combined liver and kidney transplant are 86% and 80% respectively<sup>[10]</sup>. Published series reporting the outcomes of OLT for symptomatic PLD are summarized in Table 9.

## HEPATIC RESECTION VS LIVER TRANSPLANTATION

The clinical decision between performing a hepatic resection with or without cyst fenestration<sup>[93]</sup> and referring

the patient for OLT can be extremely difficult (Table 10). Hepatic resection with cyst fenestration implies leaving residual hepatic cysts that will eventually progress<sup>[94]</sup>. However, hepatic resection is associated with a lower risk of perioperative morbidity and mortality. OLT provides the only option for the cure of these patients but requires lifelong immunosuppression and has higher perioperative risks. Both resection and OLT are technically demanding, and peri-operative care can be complex. The risks and the benefits of each of the possible treatment options have to be carefully evaluated and put in the context of the clinical presentation and condition of each patient. Referral to a tertiary center with an experienced team of surgeons, hepatologists, and nephrologists is strongly recommended.

## CONCLUSION

For patients with PLD, patients' selection, timing and choice of treatments can be very challenging even for experienced physicians. For symptomatic patients, treatment strategies should be based on the degree and progression of their symptoms and the severity of other medical conditions. Symptomatic patients with large cysts or limited hepatic involvement might benefit from fenestration or sclerotherapy. Hepatic resection with or without fenestra-

tion should be favored in patients with diffuse involvement of the liver but with sufficient spared parenchyma. Finally, in the patient with diffuse disease, OLT is a valid option and should be pursued as primary therapy prior to the development of debilitating disease such as malnutrition and liver dysfunction that can significantly increase the risks of perioperative adverse events.

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## Overlap of functional heartburn and gastroesophageal reflux disease with irritable bowel syndrome

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### Abstract

Several studies indicate a significant degree of overlap between irritable bowel syndrome (IBS) and gastroesophageal reflux disease (GERD). Likewise, both functional heartburn (FH) and IBS are functional digestive disorders that may occur in the same patients. However, data establishing a solid link between FH and IBS are lacking, mainly because the clinical definition of FH has undergone substantial changes over the years. The available literature on the overlap between GERD or FH and IBS highlights considerable heterogeneity in terms of the criteria and diagnostic procedures used to assess heartburn and IBS. In particular, several epidemiological studies included patients with concomitant IBS and GERD without any attempt to distinguish FH (as defined by the Rome III criteria) from GERD *via* pathophysiological investigations. Independent of these critical issues, there is preliminary evidence supporting a significant

degree of FH-IBS overlap. This underscores the need for studies based on updated diagnostic criteria and accurate pathophysiological classifications, particularly to distinguish FH from GERD. This distinction would represent an essential starting point to achieving a better understanding of pathophysiology in the subclasses of patients with GERD and FH and properly assessing the different degrees of overlap between IBS and the subcategories of heartburn. The present review article intends to appraise and critically discuss current evidence supporting a possible concomitance of GERD or FH with IBS in the same patients and to highlight the pathophysiological relationships between these disorders.

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**Key words:** Functional gastrointestinal disorders; Gastroesophageal reflux disease/Gastro-oesophageal reflux disease; Irritable bowel syndrome; Acidity (esophageal); Hypersensitivity

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### INTRODUCTION

Gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS) are gastrointestinal disorders that affect a large portion of the general population and have a relevant impact on quality of life and health care costs. Although these disturbances affect different regions of the digestive tract, it has been noted that they may occur in the same patient. In addition, recent studies have shown a concomitance between functional heartburn

(FH) and IBS. This finding is not completely unexpected because FH and IBS are both functional digestive disorders (FDDs), and the possibility of an overlap among different FDDs has been largely acknowledged<sup>[1]</sup>. Indeed, there is mounting evidence that FDDs consist of a number of heterogeneous syndromes characterized by various gastrointestinal symptoms with no evident organic cause found upon clinical investigation<sup>[2]</sup>.

Based on the above considerations, the hypothesis of an association between FH and IBS deserves careful attention and investigation. However, data establishing a solid link between FH and IBS are lacking, most likely because the disorders' clinical definitions have undergone to significant variations over the years, and their pathophysiology remains poorly understood.

The present article intends to provide a review of current evidence supporting a possible clinical and pathophysiological relationship between GERD/FH and IBS.

## DEFINITIONS

To properly address the relationship between GERD/FH and IBS, it is important to preliminarily clarify some definitions of GERD, as patients affected by FH have been often included in this category in both past and recent clinical investigations.

### GERD

GERD develops when the reflux of gastric contents into the esophagus leads to troublesome symptoms, with or without mucosal damage and/or complications<sup>[3]</sup>. A subcategory of GERD patients that displays reflux-related symptoms in the absence of erosive esophagitis at endoscopy is considered to have non-erosive reflux disease (NERD)<sup>[3]</sup>. Pathophysiological studies conducted *via* pH monitoring and, more recently, impedance-pH monitoring (MII-pH) have demonstrated that there are two main types of NERD patients: those with abnormal acid reflux and those with physiological acid exposure time (AET). In the latter group, patients showing a close temporal relationship between symptoms and acid or non-acid reflux episodes have been defined as having a "hypersensitive esophagus" and should be considered within the spectrum of GERD<sup>[4,5]</sup>. When the association between symptoms and physiological reflux is lacking, patients can be classified as having FH, which is defined in the next section.

### FH

The Rome II criteria for functional esophageal disorders defined FH as an episodic retrosternal burning in the absence of pathological gastroesophageal reflux, pathology-based motility disorders, or structural alterations<sup>[6]</sup>. In 2006, the Rome III committee modified the definition of FH as the occurrence of chronic retrosternal burning in the absence of either GERD or histopathology-based esophageal motility disorders. In particular, according to Rome III criteria, heartburn should be reported as hav-

ing persisted over the previous 3 mo, with a symptom onset dating to at least 6 mo before the diagnosis<sup>[7]</sup>. To exclude GERD, patients must undergo upper digestive endoscopy; in the absence of esophagitis, ambulatory pH monitoring should also be performed<sup>[4]</sup>. A lack of correspondence between symptoms and reflux episodes, together with normal acid exposure in the distal esophagus, would suggest a diagnosis of FH. Such a diagnosis could be further substantiated by the outcome of a therapeutic trial with a proton pump inhibitor (PPI); although it is not specific, an unsatisfactory response to acid inhibition is likely to have a negative predictive value in support of GERD<sup>[8]</sup>.

A recent study suggested that, to be diagnosed with FH, patients should have a normal upper endoscopy, a normal AET in the distal esophagus and a negative symptom association with both acid and non-acid reflux<sup>[5]</sup>.

The evaluation of the latter condition is possible only with MII-pH monitoring, which is able to recognize both acid and non-acid reflux. However, it must be considered that, to date, the exact role of non-acid reflux in the pathophysiology of symptoms in untreated GERD patients has been minimally evaluated. Therefore, the findings reported by Savarino *et al.*<sup>[5]</sup> should be viewed as preliminary in nature and should be substantiated by further studies before undergoing a critical assessment by consensus committees.

### IBS

According to the Rome III criteria, IBS is a functional bowel disorder in which recurrent abdominal pain or discomfort is associated with defecation and/or changes in bowel habits. In particular, abdominal pain or discomfort is associated with two or more of the following characteristics: improvement with defecation and onset associated with a change in the frequency and/or form of stool. The predominant stool pattern allows the classification of IBS into four clinical variants: with constipation; with diarrhea; mixed; and unsubtyped<sup>[9]</sup>.

## GERD/FH AND IBS OVERLAP

To date, several studies have reported a certain degree of overlap between GERD and IBS that cannot be explained solely by chance<sup>[10-13]</sup>. By contrast, epidemiological data regarding the possible concomitance of FH and IBS in the same patient are lacking.

In the last two decades, the assessment of the epidemiological and clinical features of IBS has gained considerable attention. At present, the overall prevalence of IBS ranges from 10% to 20% of adults and adolescents, and it predominantly affects young (20-45 years old) females<sup>[14,15]</sup>. Population-based studies suggest that GERD, defined by at least weekly heartburn and/or regurgitation, is a common condition, with a prevalence of 10%-20% in Western populations<sup>[16]</sup>. Several studies have shown that up to 70% of patients complaining of heartburn have NERD; 30%-50% of NERD patients display nor-

mal 24-h esophageal pH monitoring<sup>[17]</sup>, and approximately 60% of these patients show a negative relationship between symptoms and acid reflux events<sup>[4]</sup>. More recent studies conducted with MII-pH in NERD patients suggest an FH prevalence ranging from 19% to 26%<sup>[5]</sup>. Very little is currently known about gender prevalence among patients with FH, although the condition seems to be more common in women<sup>[18]</sup>.

The identification of a clinical overlap between FH and IBS is complicated by the fact that most studies have usually evaluated the concomitance of IBS and heartburn, irrespective of whether the latter was related to GERD or FH. In particular, most data have been collected *via* epidemiological studies conducted using validated questionnaires and endoscopy, without any pathophysiological attempt to discriminate GERD patients from FH patients. In this context, we were interested in performing an in-depth analysis of the overlap between GERD/FH and IBS by conducting a search of the available literature.

### Literature search

We identified the published studies to include in our review *via* an electronic search of three bibliographical databases: PubMed (1966-2011), EMBASE (1980-2011) and the Cochrane Library (2000-2011). Only studies that were designed as randomized-controlled, cross-sectional and case-control were included in our analysis. The search was performed by two investigators using the string “(reflux OR heartburn OR GERD OR GORD OR gastroesophageal reflux OR PPI OR 24-h pH) AND (IBS)”. A restriction was placed to collect articles in English only. The initial search yielded 371 titles of studies that were published as either full text papers or abstracts of scientific meetings, and all of the studies were screened by all authors to determine their eligibility. Based on our inclusion criteria, we selected 45 studies, which were used for an in-depth analysis of the prevalence of GERD/FH in patients with IBS and vice versa. In addition, the criteria and diagnostic procedures used to assess the presence of heartburn and IBS were recorded.

### Prevalence of GERD/FH in patients with IBS

Twenty-three studies evaluated the prevalence of GERD/FH in subjects with a previous diagnosis of IBS<sup>[10,12,19-39]</sup>. The details are shown in Table 1. The overall mean prevalence of GERD was 37.5%, although there was remarkable variability, with values ranging from 11% to 79%. Five studies assessed IBS according to the Manning criteria, 4 studies according to the Rome I criteria, 8 studies according to the Rome II criteria, and 6 studies according to the Rome III criteria. In 18 studies, IBS was diagnosed *via* a symptom questionnaire; in 4 studies, organic diseases were excluded with imaging techniques and laboratory tests; in 1 study, only laboratory tests were performed. In comparison, GERD was diagnosed *via* a symptom questionnaire in 18 studies and a symptom questionnaire combined with upper endoscopy in 3 studies. In 2 studies, pathophysiological evaluations

*via* esophageal manometry and pH-metry/MII-pH were performed in addition to the symptom questionnaire and upper endoscopy<sup>[20,39]</sup>. Overall, in patients with IBS, NERD was slightly more prevalent (42%) than erosive reflux disease (ERD, 38%). One study conducted in accordance with Rome III criteria estimated an FH prevalence of 59% among patients with IBS<sup>[39]</sup>.

### Prevalence of IBS in patients with GERD/FH

Thirty-two articles investigated the prevalence of IBS in subjects with a previous diagnosis of GERD/FH<sup>[10,12,23,25,26,31,32,35,36,38-60]</sup>. The details are shown in Table 2. In GERD patients, the overall mean prevalence of IBS was 36%, although there was considerable variability, as shown by values ranging from 8% to 71%. In 3 studies, IBS was diagnosed according to the Manning criteria (mean prevalence: 34.4%); in 8 studies, it was diagnosed according to the Rome I criteria (mean prevalence: 41.4%); in 10 studies, it was diagnosed according to the Rome II criteria (mean prevalence: 38.1%); in 8 studies, it was diagnosed according to the Rome III criteria (mean prevalence: 31.9%); in 3 studies according to the ReQuest criteria (mean prevalence: 37.3%). In all studies, IBS was diagnosed *via* a symptom questionnaire. However, in one study, hematological and stool examinations were also performed to exclude organic diseases<sup>[26]</sup>. In comparison, GERD was diagnosed *via* a symptom questionnaire in 18 studies and *via* a symptom questionnaire combined with upper endoscopy in 7 studies. In 7 studies, esophageal pathophysiological studies (*i.e.*, manometry and pH-metry) were performed in addition to the symptom questionnaire and upper endoscopy. Overall, IBS was more prevalent in patients with NERD (41%) than in those with ERD (23.9%). Two studies, which evaluated FH in accordance with the Rome III criteria, estimated prevalences of 39%<sup>[56]</sup> and 61.4% for IBS<sup>[39]</sup>. In the first study, heartburn was investigated *via* pH-metry, while the latter used MII-pH testing.

### Discussion

Large population-based studies have used validated questionnaires to investigate a possible association between GERD and IBS and have suggested that GERD can affect a considerable proportion of patients with IBS<sup>[22,27,28]</sup> or vice versa<sup>[43,49]</sup>. However, few studies specifically address the issue of overlap between FH and IBS, mainly because the definition of FH has varied substantially throughout the years. Indeed, the definition of FH has been greatly modified from the Rome II criteria (in which the definition of FH included all NERD patients with negative pH-metry) to the Rome III criteria (in which FH is defined as a functional esophageal disorder unrelated to GERD and characterized by negative pH-metry, the lack of a relationship between symptoms and reflux events, and the lack of symptom improvement after a trial of PPI therapy).

Notably, most of the available data on the association between IBS and GERD were collected in the context

**Table 1** Prevalence of gastroesophageal reflux disease/functional heartburn in irritable bowel syndrome patients

IBS patients (n)	IBS criteria	Diagnostic investigations of IBS	GERD prevalence	FH prevalence	Diagnostic investigations of heartburn	Ref.
101	Manning	S, HE, Sg, BE, BT, UE, SBB, BC, LE	25%	Not evaluated	SQ	Svedlund <i>et al</i> <sup>[19]</sup>
25	Manning	S, Sg, SC, HE, BE	28% (daily) 52% (weekly)	Not evaluated	S, UE, OM, pH (wireless)	Smart <i>et al</i> <sup>[20]</sup>
100	Manning	S, LE, HE, BE	30%	Not evaluated	SQ	Whorwell <i>et al</i> <sup>[21]</sup>
350	Modified manning	SQ	79%	Not evaluated	SQ	Jones <i>et al</i> <sup>[22]</sup>
546	Modified manning	Postal SQ	46.5%	Not evaluated	Postal SQ	Kennedy <i>et al</i> <sup>[23]</sup>
146	Rome I	S, PE, AU, HE, UE or BE (patients older than 50 yr)	28%	Not evaluated	S, UE	Stanghellini <i>et al</i> <sup>[24]</sup>
68	Rome I	SQ	3%	Not evaluated	SQ	Hu <i>et al</i> <sup>[25]</sup>
68	Rome I	Phone SQ	11%	Not evaluated	Phone SQ	Cheung <i>et al</i> <sup>[12]</sup>
52	Rome I	S, SC, HE	38% (ERD) 42% (NERD)	Not evaluated	S, UE	Camacho <i>et al</i> <sup>[26]</sup>
76 (IBS-C)	Rome II	SQ	32.9%	Not evaluated	SQ	Talley <i>et al</i> <sup>[27]</sup>
45 (IBS-D)	Rome II	Phone SQ	40.9%	Not evaluated	Phone SQ	Hungin <i>et al</i> <sup>[28]</sup>
3880	Rome I Manning	SQ	21%	Not evaluated	SQ	Si <i>et al</i> <sup>[29]</sup>
662	Rome II	SQ	25%	Not evaluated	SQ	Balboa <i>et al</i> <sup>[30]</sup>
517	Rome II	SQ	40%	Not evaluated	SQ	Lee <i>et al</i> <sup>[31]</sup>
95	Rome II	SQ	21%	Not evaluated	SQ	Hori <i>et al</i> <sup>[32]</sup>
40	Rome II	SQ	20%	Not evaluated	SQ	Johansson <i>et al</i> <sup>[33]</sup>
164	Rome II	SQ	43%	Not evaluated	SQ	Schmulson <i>et al</i> <sup>[34]</sup>
113	Rome II	SQ	49.6%	Not evaluated	SQ	Jung <i>et al</i> <sup>[10]</sup>
252	Rome III	Postal SQ	32.9%	Not evaluated	Postal SQ	Yarandi <i>et al</i> <sup>[35]</sup>
1419	Rome III Rome II	SQ	63.6%	Not evaluated	S, UE	Kaji <i>et al</i> <sup>[36]</sup>
381	Rome III	SQ	16%	Not evaluated	SQ	Olafsdottir <i>et al</i> <sup>[37]</sup>
1336 (in 1996)	Rome III	Postal SQ	60.5%-71.9%	Not evaluated	Postal SQ	
799 (in 2006)	Rome II Manning	SQ		Not evaluated	SQ	
381	Rome III	SQ	16%	Not evaluated	SQ	Fujiwara <i>et al</i> <sup>[38]</sup>
46	Rome III	SQ	41.3%	59%	S, UE, OM22, MII-pH	Martinucci <i>et al</i> <sup>[39]</sup>

<sup>1</sup>Articles listed in both Tables 1 and 2; <sup>2</sup>Abstract only (publication type). GERD: Gastroesophageal reflux disease; FH: Functional heartburn; IBS: Irritable bowel syndrome; S: Symptoms; SQ: Symptom questionnaire; PE: Physical examination; HE: Hematological examinations; BE: Barium enema; BC: Bacteriological culture; SC: Stool culture; BT: Lactose/lactulose breath test; AU: Abdominal ultrasonography; UE: Upper endoscopy; LE: Lower endoscopy; SBB: Small-bowel biopsies; Sg: Sigmoidoscopy; OM: Esophageal manometry; pH: pH-metry; MII-pH: pH impedance monitoring; ERD: Erosive reflux disease; NERD: Nonerosive reflux disease.

of epidemiological studies, which were conducted on patients with heartburn using validated questionnaires and upper endoscopy without the use of any reliable pathophysiological investigation to discriminate FH (according to the Rome III criteria) from GERD.

As mentioned above, only two studies have evaluated the concomitance of FH and IBS. Lee *et al*<sup>[56]</sup> examined 95 patients with heartburn by endoscopy, pH-metry, PPI test, and psychological characteristics. The patients were classified using the Rome III criteria; therefore, FH was diagnosed based on physiological AET, a negative association between symptoms and reflux, and a negative PPI test in patients without erosive esophagitis. A higher prevalence of IBS was recorded in FH patients (39%) than in ERD (17%) or NERD (23%) patients. Furthermore, anxiety was more prevalent in FH patients than in NERD patients. Recently, we examined 92 patients with heartburn (without esophageal mucosal breaks found upon upper endoscopy) *via* pH-MII to assess, in accordance with Rome III criteria, the prevalence of NERD

subgroups and FH in two groups of patients: those with and those without IBS. For each subject, we evaluated the AET, number of reflux episodes, correlation between symptoms and refluxes, and subjective response to PPI therapy. FH was found in 59% (27/46) of the patients with IBS, compared with 37% (17/46) of the patients without IBS ( $P < 0.05$ ), indicating a higher prevalence of FH in IBS patients. In comparison, IBS was found in 39.6% (19/48) of the patients with NERD and in 61.4% (27/44) of the patients with FH, suggesting that in IBS patients, FH was more common than NERD was<sup>[39]</sup>. Although data from these two pioneering studies are not sufficient to support the concept that FH and IBS can occur in the same patient, they underscore the need for future investigations based on updated diagnostic criteria.

## PATHOPHYSIOLOGICAL SIMILARITIES IN GERD, FH AND IBS

Previous studies dealing with the overlap between GERD

**Table 2** Prevalence of irritable bowel syndrome in gastroesophageal reflux disease/functional heartburn patients

GERD patients (n)	FH patients (n)	Diagnostic investigations of heartburn	IBS prevalence	IBS criteria	Diagnostic investigations of IBS	Authors
910	Not evaluated	Postal SQ	19%	Manning	Postal SQ	Kennedy <i>et al</i> <sup>[23]1</sup>
80	Not evaluated	SQ	36.7%-45.1%	Manning	SQ	Chey <i>et al</i> <sup>[40]2</sup>
34 (ERD)	Not evaluated	S, UE	36% (in ERD)	Manning	SQ	Nojkov <i>et al</i> <sup>[41]</sup>
67 (NERD)			35% (in NERD)			
643	Not evaluated	SQ	42%	Rome I	SQ	Locke <i>et al</i> <sup>[42]</sup>
35	Not evaluated	SQ	71%	Rome I	SQ	Pimentel <i>et al</i> <sup>[43]</sup>
79	Not evaluated	SQ	3%	Rome I	SQ	Hu <i>et al</i> <sup>[25]1</sup>
457	Excluded	S, UE, OM, pH	49%	Rome I	SQ	Zimmerman <i>et al</i> <sup>[44]</sup>
79	Not evaluated	Phone SQ	13%	Rome I	Phone SQ	Cheung <i>et al</i> <sup>[11]21</sup>
326 (NERD)	Excluded	S, UE, pH	48.5%	Rome I	SQ	Hershovici <i>et al</i> <sup>[45]</sup>
326 (NERD)	Excluded	S, UE, pH	49%	Rome I	SQ	Zimmerman <i>et al</i> <sup>[46]</sup>
41 (ERD)	Not evaluated	S, UE	48.7% (in ERD)	Rome I	S, SC, HE	Camacho <i>et al</i> <sup>[26]1</sup>
45 (NERD)			48.8% (in NERD)			
3318	Not evaluated	SQ	36.7%-45.1%	Rome II	SQ	Bueno <i>et al</i> <sup>[47]2</sup>
102	Excluded	S, UE, OM, pH	32.4%	Rome II	SQ	Raftopoulos <i>et al</i> <sup>[48]</sup>
3318	Not evaluated	SQ	27%	Rome II	SQ	Guillemot <i>et al</i> <sup>[49]</sup>
263	Not evaluated	S, pH	35%	Rome II	SQ	De Vries <i>et al</i> <sup>[50]</sup>
111 (ERD)	Excluded	S, UE, OM, pH	15.3% (in ERD)	Rome II	SQ	Wu <i>et al</i> <sup>[51]</sup>
113 (NERD)			44.2% (in NERD)			
238	Not evaluated	SQ	60.9%	Rome II	SQ	Nasseri-Moghaddam <i>et al</i> <sup>[52]</sup>
67	Not evaluated	SQ	27%	Rome II	SQ	Lee <i>et al</i> <sup>[31]1</sup>
16	Not evaluated	SQ	50%	Rome II	SQ	Hori <i>et al</i> <sup>[32]1</sup>
92	Not evaluated	SQ	62%	Rome II	SQ	Rey <i>et al</i> <sup>[53]</sup>
102 (ERD)	Excluded	S, UE, OM, pH	20.6% (in ERD)	Rome II	SQ	Wu <i>et al</i> <sup>[54]</sup>
163 (NERD)			39.9% (in NERD)			
411	Not evaluated	Postal SQ	20.2%	Rome III	Postal SQ	Jung <i>et al</i> <sup>[10]1</sup>
344	Not evaluated	SQ	51.7%	Rome III	SQ	Solhpour <i>et al</i> <sup>[55]</sup>
36/95 (ERD)	23/95	S, UE, OM, pH	17% (in ERD)	Rome III	SQ	Lee <i>et al</i> <sup>[56]</sup>
36/95 (NERD)			23% (in NERD)			
			39% (in FH)			
207	Not evaluated	SQ	29.5%	Rome III	SQ	Kaji <i>et al</i> <sup>[36]1</sup>
286 (ERD)	Not evaluated	S, UE	11.2%	Rome III	SQ	Noh <i>et al</i> <sup>[37]</sup>
74 (NERD)			41.9%			
2658	Not evaluated	S, UE	33.9%	Rome III	SQ	Yarandi <i>et al</i> <sup>[35]1</sup>
				Rome II		
207	Not evaluated	SQ	29.5%	Rome III	SQ	Fujiwara <i>et al</i> <sup>[38]1</sup>
48/92 (NERD)	44/92	S, UE, OM22, MII-pH	39.6% (in NERD)	Rome III	SQ	Martinucci <i>et al</i> <sup>[39]1,2</sup>
			61.4% (in FH)			
1181 (ERD)	Not evaluated	S, UE	12.7% (in ERD)	ReQuest	SQ	Mönnikes <i>et al</i> <sup>[58]</sup>
694 (NERD)			18.3% (in NERD)			
6810	Not evaluated	SQ	60%	ReQuest	SQ	Fass <i>et al</i> <sup>[59]2</sup>
257	Not evaluated	SQ	58%	ReQuest	SQ	Bardhan <i>et al</i> <sup>[60]</sup>

<sup>1</sup>Articles listed in both tables 1 and 2; <sup>2</sup>Abstract only (publication type). GERD: Gastroesophageal reflux disease; FH: Functional heartburn; IBS: Irritable bowel syndrome; S: Symptoms; SQ: Symptom questionnaire; PE: Physical examination; HE: Hematological examinations; BE: Barium enema; BC: Bacteriological culture; SC: Stool culture; BT: Lactose/lactulose breath test; AU: Abdominal ultrasonography; UE: Upper endoscopy; LE: Lower endoscopy; LEB: Lower endoscopy and biopsies; SBB: Small-bowel biopsies; Sg: Sigmoidoscopy; OM: Esophageal manometry; pH: pH-metry; MII-pH: pH impedance monitoring; ERD: Erosive reflux disease; NERD: Nonerosive reflux disease.

and IBS have proposed that visceral hypersensitivity, motility dysfunctions, and central neural mechanisms can be the main common pathophysiological mechanisms<sup>[11,13,61]</sup>. However, following the release of Rome III criteria, an increasing number of studies have indicated the importance of a careful categorization of GERD patients *via* pathophysiological investigations to better appreciate the degrees of overlap between IBS and reflux symptoms in various subgroups of patients<sup>[39,56,62,63]</sup>. Accordingly, this section intends to appraise and critically discuss the available evidence supporting a pathophysiological relationship among GERD, FH and IBS. When attempting such a difficult task, two important points must be care-

fully considered: (1) In previous studies, GERD and IBS patients have been investigated to determine their pathophysiological and clinical features, while FH patients constitute a “new entity” for which pathophysiological studies are urgently required; and (2) Most of the available literature on the pathophysiology of FH addresses patients who were identified using old criteria (*i.e.*, criteria that have since been replaced by the Rome III classification) that also identified NERD patients with normal esophageal AET. Even when these issues are kept in mind, IBS and FH, as well as IBS and GERD, appear to share some pathophysiological features that need to be carefully considered.

### Visceral hypersensitivity

Most FDD patients display a reduced pain or discomfort threshold in response to visceral stimulation, implying that they might perceive a stimulus as uncomfortable or painful at significantly lower intensity than normal subjects would<sup>[64]</sup>. Such increased sensitivity can be usually documented throughout the whole gastrointestinal tract, suggesting diffuse, rather than site-dependent, involvement<sup>[65]</sup>.

Studies aimed at gaining pathophysiological insights irrespective of the dominant digestive disorder have extensively investigated visceral hypersensitivity to a variety of stimuli (*e.g.*, acid perfusion, balloon distension, electrical stimulation) within both IBS<sup>[66]</sup> and GERD<sup>[63]</sup>. In particular, current data suggest that NERD patients displays equivalent or increased degrees of visceral hypersensitivity as compared with ERD, but may have lower levels than those shown by patients with functional esophageal disorders (*i.e.*, FH/chest pain of presumed esophageal origin). According to recent advances in basic science, three main mechanisms are believed to underlie visceral hypersensitivity (*i.e.*, peripheral sensitization, central sensitization and psychoneuroimmune interactions), and all of these have been documented in NERD patients<sup>[63]</sup>. Nevertheless, these factors' respective roles and degrees of involvement in the pathophysiology of FH remain to be established, particularly in the light of the Rome III criteria. To verify whether FH patients have visceral hypersensitivity and to assess whether this feature is a common trait in IBS patients, some studies have investigated the presence of esophageal sensitivity to chemical or mechanical stimuli in FH and/or IBS patients.

Rodriguez-Stanley *et al.*<sup>[67]</sup> reported that 89% of patients with FH (Rome II) experienced abnormal responses to intraesophageal acid perfusion (Bernstein test), esophageal balloon distension, or both. In repeated studies using either esophageal balloon distension or electrical stimulation, patients with FH (Rome II) have consistently demonstrated a lower perception threshold for pain or discomfort compared with patients with erosive esophagitis and/or abnormal 24-h esophageal pH monitoring<sup>[68,69]</sup>. Recently, Thoua *et al.*<sup>[62]</sup> observed that patients with NERD had higher sensitivity to esophageal acid exposure than did ERD patients and controls, and this hypersensitivity was most pronounced with proximal esophageal acid exposure. Moreover, FH patients (Rome III) were more hypersensitive to excess acid exposure than NERD patients were. Of note, these authors carefully selected patients with unequivocal reflux, taking care to exclude those with minor mucosal breaks, and the condition of hypersensitivity was found to be independent from motility changes<sup>[62]</sup>. Yang *et al.*<sup>[70]</sup> found that cortical evoked potentials latencies induced by balloon distension were shorter in FH patients (Rome II) than in controls before acid perfusion, and such perfusion decreased the latencies and increased their amplitude in FH patients, but not in controls. These findings suggest that dysfunctions of visceral neural pathways and/or alterations in cortical processing might generate and mediate esophageal hypersensitivity in FH.

Consistent with the notion that visceral hypersensitivity is not site-specific, Costantini *et al.*<sup>[71]</sup> reported that during esophageal provocative testing (balloon distension and bethanechol administration), IBS patients displayed a lower threshold for esophageal symptoms compared with healthy volunteers, without any evident alteration of esophageal motility or decrease in esophageal basal pressure. In line with these observations, Trimble *et al.*<sup>[72]</sup> demonstrated that IBS patients had a lower rectal sensory threshold for pain compared with healthy controls and that IBS patients displayed concomitantly lower sensory thresholds for both esophageal perception and discomfort evoked by balloon distension.

Whether the types of sensory dysfunctions previously detected in FH patients (Rome II)<sup>[68]</sup> can also be observed in FH patients diagnosed in accordance with Rome III criteria remains to be established. When investigating this issue, it must be considered that at present, there is not a unanimous consensus on how to define and measure the condition of lowered visceral threshold. A further critical issue is that visceral thresholds for different stimuli do not necessarily display parallel alterations. In this context, some relevant questions still await conclusive answers: (1) Which is the most meaningful index of an altered sensory threshold? (2) Can different stimuli be regarded as equivalent in nature? and (3) Considering day-to-day variations in the occurrence of symptoms, is there also a day-to-day variation in the underlying biological abnormalities responsible for these symptoms? Overall, great caution will be required in future studies addressing the pathophysiological meaning of visceral hypersensitivity in GERD/FH and/or IBS.

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### Motility dysfunction

Motor abnormalities might represent a common pathophysiological mechanism between GERD and IBS<sup>[61]</sup>. Consistent with this concept, some authors speculate that an overall dysfunction of smooth muscle throughout the GI tract might explain the overlap between IBS and GERD<sup>[22]</sup>.

Of note, the pattern of esophageal motility has been shown to differ between ERD and NERD patients<sup>[73]</sup>, while no significant differences have been found in LES pressure or contraction amplitude when comparing FH patients (Rome III) to NERD patients with pathological AET<sup>[62]</sup>. In unclassified subjects complaining of heartburn, Bhalla *et al.*<sup>[74]</sup> observed that acid infusion elicited an increase in symptom sensitivity in concomitance with a perturbation of esophageal contractility, as revealed by a greater increase in contraction amplitude, contraction duration, muscle thickness, and the incidence of sustained esophageal contractions during the second acid infusion in comparison with the first one.

To date, the possible contribution of motility dysfunction to the pathophysiology of FH remains unclear; however, while studying 12 unclassified subjects with heartburn using 24-h pH-metry, synchronized pressure

recording and high-frequency intraluminal ultrasound imaging of the oesophagus, Pehlivanov *et al.*<sup>[75]</sup> highlighted a close correlation between heartburn episodes (whether associated with acid reflux or not) and abnormally long longitudinal muscle contraction durations. This motor correlate might also be relevant to a better understanding of the pathophysiological bases of heartburn perception in FH patients, but it has been documented only by a preliminary investigation and requires additional studies to be confirmed. Likewise, whether esophageal and bowel motor abnormalities occur concomitantly in patients with overlapping GERD/FH and IBS is currently unclear, and studies addressing this issue are required.

### Central neural mechanisms

In FH patients, heartburn has been proposed to originate from factors other than luminal stimuli<sup>[68]</sup>. It has been speculated that central neural mechanisms related to psychological comorbidity (anxiety, depression and stress) could modulate esophageal perception and make patients prone to perceiving low-intensity esophageal stimuli as painful<sup>[69]</sup>. In particular, anxiety has been implicated as a factor that may modulate the degree of sensitization to esophageal acid testing<sup>[76]</sup>.

Johnston *et al.*<sup>[77]</sup> studied 101 patients with heartburn using esophageal pH monitoring. The subjects who showed no correlation between symptoms and refluxes displayed significantly higher levels of trait anxiety compared with patients with a positive correlation. Along the same line, Rubenstein *et al.*<sup>[78]</sup> observed that in subjects with heartburn, esophageal sensation to both acid perfusion and mechanical distension was associated with increased levels of psychiatric distress and a diagnosis of IBS.

According to Posserud *et al.*<sup>[79]</sup>, no clear relationship between pain threshold and IBS symptoms (severe pain, bloating and diarrhea) has been convincingly established, and other mechanisms, including central nervous ones, are likely to play a relevant role. In line with this contention, Elsenbruch *et al.*<sup>[80]</sup> observed that IBS patients can indeed experience a higher severity of distension-induced pain and overall discomfort despite unaltered rectal sensory thresholds, suggesting that the perception of visceral stimuli could be influenced by emotional factors. In contrast, it remains unclear what psychological factors are relevant for visceral hyperalgesia in IBS patients and how they may interact with biological mechanisms, such as peripheral/central neuroendocrine and immune processes<sup>[66]</sup>.

Another aspect that deserves attention addresses the possible impact of sleep disorders on the pathophysiology of FDD symptoms. Jung *et al.*<sup>[10]</sup> observed that self-reported insomnia and frequent abdominal pain represent two risk factors for IBS-GERD overlap compared with IBS or GERD alone. In addition, a positive association has been found between the severity of IBS symptoms and the severity of sleep disturbances. However, the pathophysiological mechanisms underlying this association are only partly understood. One possibility

is that sleep disorders induce visceral hyperalgesia, thus amplifying the patient's perception of gastrointestinal symptoms<sup>[81,82]</sup>.

### Response patterns to drugs that modulate visceral pain

Pathophysiological similarities among GERD, FH and IBS might reflect similarities in their response patterns to the drugs that influence common pathophysiological mechanisms. According to the Rome III criteria, FH patients' symptoms do not improve with PPI therapy. Consistent with this criterion, even before Rome III, some authors reported that adding or switching PPIs to a visceral pain modulator [(*i.e.*, tricyclic antidepressants (TCAs) or selective serotonin reuptake inhibitors (SSRIs)] might induce beneficial effects in FH patients (Rome II)<sup>[83]</sup>. Peghini *et al.*<sup>[84]</sup> were the first to report that imipramine can reduce esophageal pain perception in healthy male volunteers. Clouse *et al.*<sup>[85]</sup> investigated the effects of low-dose trazodone in patients with symptomatic esophageal dysmotility and obtained a significantly greater global symptom improvement compared with placebo. Broekaert *et al.*<sup>[86]</sup> observed that citalopram lowered chemical and mechanical esophageal sensitivity in healthy subjects without altering motility. Likewise, in a randomized placebo-controlled study, citalopram 20 mg/d was found to be effective in a selected group of patients with hypersensitive esophagus (*i.e.*, normal AET, positive SI)<sup>[87]</sup>. Overall, the current evidence, although preliminary in nature, suggests that SSRIs may exert beneficial effects in lowering esophageal sensitivity to chemical and mechanical stimuli. These observations encourage the performance of studies aimed at assessing the efficacy of SSRIs in patients with esophageal hypersensitivity. In this regard, it is interesting to note that antidepressants (*e.g.*, TCAs and SSRIs) have been found more effective than placebo in IBS treatment, as indicated by a recent review and meta-analysis of randomized controlled trials<sup>[88]</sup>. Thus, based on current knowledge, it can be tentatively speculated that visceral hypersensitivity might be a common trait among patients with esophageal hypersensitivity and/or IBS and that such an underlying pathophysiological condition might explain the beneficial responses to antidepressants in both these disorders. Overall, a critical appraisal of current evidence highlights the need for future clinical studies aimed at assessing the possible transverse beneficial actions of drugs in patients with concomitant ERD, NERD or FH and IBS. To date, it can be hypothesized that antidepressants have a beneficial role as visceral pain modulators.

## CONCLUSION

In the present review, we have attempted to appraise and critically discuss whether the current literature supports an association between GERD and IBS and between FH and IBS. Our literature search highlights a high heterogeneity in terms of both the criteria and diagnostic procedures used to investigate the presence of heartburn

and IBS. In particular, most of the current epidemiological data do not rely on a formal diagnostic assessment of IBS and/or GERD; rather, the studies generally evaluated these disorders *via* symptom questionnaires. Another critical issue is the inclusion of patients with concomitant IBS and GERD without any attempt to distinguish FH from GERD using pathophysiological investigations. Indeed, a very few small studies have documented an actual concomitance of FH and IBS. The main reason for this paucity of data stems from the fact that, until the release of the Rome III criteria, FH was not regarded as a distinct entity and was included in the same category as GERD. Moreover, most of current pathophysiological data refer to FH patients as defined by criteria older than the Rome III classification. Accordingly, clear evidence of an association between IBS and FH, as defined by the Rome III criteria, is presently lacking.

Independent of these critical issues, there is some evidence, though scarce and preliminary, of the concomitance of FH and IBS. In support of this contention, some studies have shown that FH and IBS may share common pathophysiological mechanisms, such as visceral hypersensitivity, and that drugs that act as visceral pain modulators (such as antidepressants) may exert beneficial effects on both disorders when tested in separate trials.

Overall, current knowledge about the GERD/FH and IBS overlap needs to be expanded *via* investigations based on updated diagnostic criteria, more accurate pathophysiological classifications, and careful categorization of patients with heartburn. To achieve these goals, future epidemiological and pathophysiological studies should be designed to properly assess the presence and extent of overlaps linking IBS with FH and various subgroups of GERD patients. In this context, it is also expected that a better pathophysiological characterization of heartburn will foster the identification of therapeutic strategies that target the common pathogenic mechanisms underlying FH and IBS.

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## Criteria for the diagnosis and severity stratification of acute pancreatitis

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### Abstract

Recent diagnostic and therapeutic progress for severe acute pancreatitis (SAP) remarkably decreased the case-mortality rate. To further decrease the mortality rate of SAP, it is important to precisely evaluate the severity at an early stage, and initiate appropriate treatment as early as possible. Research Committee of Intractable Diseases of the Pancreas in Japan developed simpler criteria combining routinely available data with clinical signs. Severity can be evaluated by laboratory examinations or by clinical signs, reducing the defect values of the severity factors. Moreover, the severity criteria considered laboratory/clinical severity scores and contrast-enhanced computed tomography (CE-CT) findings as independent risk factors. Thus, CE-CT scans are not necessarily required to evaluate the severity of acute pancreatitis. There was no fatal case in mild AP diagnosed by the CE-CT severity score, whereas case-mortality rate in those with SAP was 14.8%. Case-mortality of SAP that fulfilled both the laboratory/clinical and the CE-CT severity criteria was 30.8%. It is recommended, therefore, to perform CE-CT examination to clarify the prognosis in those patients who were diagnosed as SAP by laboratory/clinical severity criteria. Because the mortality rate of these patients with SAP is high, such patients should be transferred to advanced medical units.

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**Key words:** Severe acute pancreatitis; Severity score; Scoring system; Prognostic factors; Case-mortality

**Core tip:** The new severity criteria of acute pancreatitis (AP) consist of two independent prognostic factors; laboratory and/or clinical severity scores and contrast-enhanced computed tomography (CE-CT) findings. Mortality rate of severe acute pancreatitis (SAP) that

satisfied both laboratory/clinical and CE-CT severity criteria was as high as 30.8%. It is recommended to perform CE-CT examination in those patients who were diagnosed as SAP by laboratory/clinical severity criteria. Patients who fulfill both severity criteria should be transferred to advanced medical units. The revised criteria are extremely useful to detect SAP at an early stage of AP.

Otsuki M, Takeda K, Matsuno S, Kihara Y, Koizumi M, Hirota M, Ito T, Kataoka K, Kitagawa M, Inui K, Takeyama Y. Criteria for the diagnosis and severity stratification of acute pancreatitis. *World J Gastroenterol* 2013; 19(35): 5798-5805 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5798.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5798>

## INTRODUCTION

Acute pancreatitis (AP) involves various clinical features from mild cases with only transient abdominal symptoms to severe fatal cases. It is important to identify patients with AP who are at risk for developing persistent organ failure early in the course of the disease<sup>[1]</sup>. Because case-mortality rate of severe AP (SAP) at the survey conducted by the Research Committee of Intractable Diseases of the Pancreas (RCIDP) supported by the Japanese Ministry of Health, Labour and Welfare was as high as 30%<sup>[2]</sup>, SAP has been designated as an intractable disease by the Japanese Ministry of Health and Welfare since 1990, and the cost of treatment for SAP is paid in full by the government<sup>[3]</sup>. With the start of the medical expense payment system for patients with SAP, the RCIDP established the criteria for the diagnosis and severity stratification of AP. The severity scoring system was revised and the 2002 version was developed in 2002 (JPN criteria 2002)<sup>[1,4-6]</sup>.

The criteria 2002 were complicated and composed of 18 items of prognostic factors; 5 clinical sign items, 10 blood test items, computed tomography (CT) findings, the presence of systemic inflammatory response syndrome (SIRS) and age<sup>[1,4-6]</sup>. The attending physician cannot remember all or even most of the factors. Moreover, these numerous parameters are not available soon enough or not available as the routine laboratory tests at all hospitals. There is a possibility, therefore, that incomplete examinations or defect values of the prognostic factors underestimated the severity of AP, resulting in insufficient and inadequate treatment of the disease, and aggravated AP<sup>[1]</sup>. There is another possibility that incomplete severity evaluation of AP overlooked the predicted serious cases to transfer to medical institutions with the high-level medical facilities and intensive care.

To decrease the mortality rate of the SAP, it is important to precisely evaluate the severity early in the disease and initiate appropriate treatment as early as possible<sup>[7-9]</sup>. The Ranson<sup>[10]</sup> and the modified Glasgow (Imrie) scores<sup>[11]</sup> represent a major advantage in the evaluation of

the disease severity in AP but require 48 h of data collection before the severity can be evaluated. Thereafter, several clinical scoring systems such as acute physiology and chronic health evaluation (APACHE II) score systems<sup>[12-15]</sup>, SIRS<sup>[16]</sup>, bedside index for severity in acute pancreatitis (BISAP)<sup>[17]</sup> and harmless acute pancreatitis score (HAPS)<sup>[18,19]</sup> for evaluating AP have been developed, but these methods to predict the development of SAP are complicated, cumbersome, and insufficiently sensitive<sup>[20]</sup>. Recently a web-based consultative process involving multiple international pancreatic societies revised and updated the Atlanta classification of AP<sup>[21-23]</sup>. Severity of the disease is classified as mild, moderate, and severe by the absence or presence of organ failure and local or systemic complications. Moderately SAP has transient organ failure of < 2 d, while SAP is defined by the presence of persistent organ failure for  $\geq$  2 d. Although the revised Atlanta classification of AP is simple and will help the clinician to predict the outcome of patients with AP, it is unable to differentiate between moderately SAP and SAP before 48 h after onset. It is expected, therefore, to develop simpler severity scoring system with routinely available data that predicts outcome, the system that clinicians can use at the bedside.

## PROBLEM OF THE PREVIOUS JPN CRITERIA FOR THE DIAGNOSIS AND STRATIFICATION OF THE SEVERITY

JPN clinical criteria for the diagnosis of AP proposed in 2002 are (1) acute abdominal pain and tenderness in the upper abdomen; (2) elevated pancreatic enzyme levels in serum, urine or ascitic fluid; and (3) ultrasonographic (US) or radiologic abnormalities characteristic of AP<sup>[1,4-6]</sup>. When at least two of the above conditions are present, then excluding other pancreatic and acute abdominal diseases of different causes can make the diagnosis of AP. Acute exacerbation of chronic pancreatitis is also included in this category. When diagnosis is confirmed by surgery and/or autopsy, the event has to be duly recorded<sup>[1,4-6]</sup>.

The JPN severity criteria 2002 consisted of 5 clinical sign items (shock, respiratory failure, mental disturbance, severe infection, hemorrhagic diathesis), 10 blood test items [base excess (BE), hematocrit (Ht), blood urea nitrogen (BUN) or creatinine, calcium concentration (Ca), fasting blood glucose, arterial oxygen saturation (PaO<sub>2</sub>), lactate dehydrogenase (LDH), total protein, prothrombin time (PT), and platelet count], and CT findings. In cases with severity scores  $\geq$  2 points, SIRS and an age over 70 had to be added to the prognostic factors<sup>[1,4-6]</sup>. These items of prognostic factors were all scored as severity scores, 1 or 2 points for each positive factor, and the highest possible total score was 27 points. However, blood glucose level, serum total protein concentrations and Ht are inappropriate for the prognostic factors after the initiation of the treatment of the disease because initial fluid resuscitation might have an influence on the

measurement value of these laboratory data. In addition, the severity criteria had redundant prognostic factors indicating similar clinical condition such as shock and the decrease in BE, and dyspnea and fall of PaO<sub>2</sub>. Bleeding tendency, platelet counts and PT also indicate similar clinical condition. Moreover, the clinical signs such as severe infection that rarely develops within 48 h after disease onset were implicated in the severity criteria. The severity criteria included CT grade by the non-enhanced plain CT scan as one of the prognostic factors. Plain CT scans can evaluate peripancreatic inflammatory changes, but are unable to identify pancreatic necrosis that is closely associated with various complications and prognosis<sup>[21,24-26]</sup>.

Usefulness of the JPN criteria 2002 for severity stratification was evaluated in 1131 consecutive patients with AP that had been admitted to high specific or intensive therapy units of the affiliated research group hospitals from January 1 1995 to December 31 1998 (survey 1998; before the establishment of the JPN criteria 2002), and in 1768 patients who visited the hospitals in the year 2003 (survey 2003; from January 1 to December 31; after the establishment of the criteria in 2002)<sup>[1,4-6]</sup>. The results revealed that the severity score have almost the same value for assessment as the APACHE II score and the Ranson score<sup>[4]</sup>.

In survey 1998, case-fatality rate of mild, moderate and SAP was 0.2%, 1.6% and 13.8%, respectively, whereas it was 0.1%, 0.7% and 9.0%, respectively, in survey 2003<sup>[1,4-6]</sup>. The case-mortality rate of mild and moderate AP was quite low, and there was little clinical significance to differentiate moderate from mild AP. The case-mortality rate of SAP at stage 2 (3.7%) was low compared with that at stage 3 (25.4%) in survey 2003<sup>[1,4-6]</sup>. Therefore, it was inappropriate to classify these patients at stage 2 as SAP and identify as applicants for the medical expense payment system<sup>[5]</sup>.

Although the previous severity criteria classified prognostic factors into 2 groups; each of the items in the first group has 2 points, while that in the second group is 1<sup>[1,4-6]</sup>, there is no significant difference in case mortality between these 2 groups with different prognostic scores. JPN severity criteria 2002 were complicated and included several prognostic factors which cannot be measured at outpatient clinic or emergency room, especially at night. In addition, multiple scoring systems of the severity criteria were very cumbersome to use and they suffer from their complexity<sup>[1,4-6]</sup>. Indeed, 56% of 1768 clinical records of AP in survey 2003 had defect values of more than 3 items of 11 laboratory examinations. Especially, BE was measured in only 25.1%, and PT and PaO<sub>2</sub> were measured in only 38.3% and 38.7%, respectively<sup>[1]</sup>. These results indicate that even if we can diagnose the patient as AP, in the presence of many defect values a correct stage classification is difficult, and it is very likely that we underestimate the severity.

Because CT grade was included as one of the prognostic factors<sup>[1,4-6]</sup>, it was required to perform CT examination repeatedly to precisely evaluate the severity and

stage of AP. However, it is unacceptable to perform CT examination repeatedly<sup>[27]</sup>, and thus one of the prognostic factors remains as a defect value. In addition, there are many hospitals that cannot perform CT examination and laboratory tests such as PT, especially at night. This might be one of the reasons for many defect values in the clinical records of AP<sup>[1]</sup>.

## NEW DIAGNOSTIC CRITERIA OF AP

JPN diagnostic criteria of AP are revised taking into account of the recent progress of imaging studies and laboratory examinations of pancreatic enzymes. The revised clinical criteria for the diagnosis of AP are (1) acute pain and tenderness in the upper abdomen; (2) elevated pancreatic enzyme levels in blood and/or urine; and (3) ultrasound (US), CT or magnetic resonance imaging (MRI) abnormalities of the pancreas characteristic of AP<sup>[1]</sup>. When at least two of the above conditions are present, the diagnosis of AP can be made by excluding other pancreatic and acute abdominal diseases of other causes than pancreatitis. Acute exacerbation of chronic pancreatitis is included in this category.

Measurement of pancreatic enzyme levels in serum has been generally adopted in clinical practice, whereas those in ascitic fluid and urine are rarely determined. Since, however, recent studies have demonstrated that urinary strip tests for trypsinogen activation peptide (TAP) and trypsinogen-2 provide a reliable early diagnosis of AP<sup>[28-35]</sup>, the revised diagnostic criteria included the elevation of the pancreatic enzymes in serum and/or urine, excluding that in ascitic fluid. It is well known, however, that some patients with AP, mostly alcoholic etiology, show normoamylasemia<sup>[28]</sup>, and that serum amylase level rises only slightly in many patients with acute exacerbations of chronic alcoholic pancreatitis<sup>[36]</sup>. Moreover, serum amylase level seldom rises in AP caused by hyperlipidemia<sup>[37,38]</sup> and in those with pancreatic insufficiency<sup>[39]</sup>. In addition, the elevation of serum amylase level is only transient and declines within 3 d after onset of AP<sup>[28,40]</sup>. On the other hand, abnormally high values of serum lipase persist for longer period than that of serum amylase and are observed even in cases of alcohol-induced pancreatitis<sup>[41]</sup>. Although a recent case report of AP has demonstrated that serum amylase and lipase remain normal throughout the acute phase of AP in a man with pancreatic insufficiency and cystic fibrosis<sup>[39]</sup>, serum lipase is considered to be a more reliable diagnostic marker of AP than serum amylase. Therefore, the revised diagnostic criteria recommend determining pancreatitis specific enzymes in serum and/or urine such as pancreatic-type amylase<sup>[42,43]</sup> and lipase<sup>[44]</sup>.

The new diagnostic criteria require the presence of clear findings indicating AP by imaging studies such as US, CT and MRI. US can visualize pancreatic enlargement, inflammatory changes around the pancreas, and abnormal findings associated with AP such as the presence of ascitic fluid and gallstones. US examination can

**Table 1 Laboratory/clinical criteria for grading the severity of acute pancreatitis**

No.	Laboratory/clinical criteria
1	Base excess $\leq$ -3 mEq/L or shock (systolic blood pressure $\leq$ 80 mmHg)
2	PaO <sub>2</sub> $\leq$ 60 mmHg (room air) or respiratory failure (artificial respiratory ventilation)
3	BUN $\geq$ 40 mg/dL or creatinine $\geq$ 2.0 mg/dL or oliguria (urinary volume $\leq$ 400 mL/d after hydration)
4	LDH: More than twice higher than the upper limit of normal ( $\geq$ 700 IU/L)
5	Serum total Ca $\leq$ 7.5 mg/dL
6	Platelet count $\leq$ $1 \times 10^5$ /mm <sup>3</sup>
7	CRP $\geq$ 15 mg/dL
8	Positive score of SIRS criteria $\geq$ 3
9	Age $\geq$ 70 yr

One point for each positive factor. Severe acute pancreatitis: total scores  $\geq$  3 points. BUN: Blood urea nitrogen; LDH: Lactate dehydrogenase; SIRS: Systemic inflammatory response syndrome; CRP: C-reactive protein.

be performed repeatedly at bedside. CT provides clear local images without being affected by the adipose tissue in the abdominal wall and abdominal cavity<sup>[14,45]</sup>. CT findings of an enlarged pancreas, inflammatory changes around the pancreas and fluid collections are useful marker for the diagnosis of AP. Thus, CT is the most important imaging procedures for the diagnosis of AP<sup>[46-48]</sup>. MRI scanning can also visualize the enlargement of the pancreas and the inflammatory changes around the pancreas<sup>[49,50]</sup>.

### SEVERITY CRITERIA OF AP BY MULTIPLE-SCORING SYSTEM

Following the correct diagnosis of AP, severity stratification should be performed promptly and repeatedly, in particular for the first 48 h after the onset of the disease<sup>[1]</sup>. Early recognition of severe disease and application of appropriate therapy require vigilance as decisions regarding management need to be made shortly after admission.

The revised severity score put the redundant factors that show similar clinical conditions together into one, and deleted the unclear clinical signs. Since the new severity criteria combined laboratory data with clinical signs, the severity of AP can be evaluated by one of these findings. BE can be substituted by shock (systolic blood pressure less than 80 mmHg), PaO<sub>2</sub> by respiratory failure (artificial respiratory ventilation), and BUN or creatinine by oliguria (urinary volume less than 400 mL/d after hydration). Thus, SAP can be properly diagnosed by reducing underestimation of severity by the defect values (Table 1).

Among several serum biochemical markers that have been developed for severity stratification of AP, C-reactive protein (CRP) remains the most useful<sup>[19,32,51-54]</sup>. Although its increase delays, peaking not earlier than 72 h after the onset of symptoms, it is accurate and widely available. According to United Kingdom guidelines for the man-

**Table 2 Contrast-enhanced computed tomography criteria for grading the severity of acute pancreatitis**

Contrast-enhanced computed tomography criteria	Scores
Extension of extrapancreatic inflammatory changes	
Anterior pararenal extraperitoneal space	0 point
Root of the mesocolon	1 point
Beyond inferior renal pole	2 points
Unenhanced area in the pancreatic parenchyma (Divide the pancreas into 3 areas for expediency, head, body and tail)	
Limited to one area or peripancreatic area	0 point
Extend over 2 areas	1 point
More than 2 areas	2 points

Severe acute pancreatitis: total computed tomography severity scores  $\geq$  2 points.

agement of AP<sup>[55]</sup> and the Working Party of the Program Committee of the Bangkok World Congress of Gastroenterology 2002<sup>[56]</sup>, CRP  $\geq$  15 mg/dL is adopted as a prognostic factor. Moreover, Gardner *et al.*<sup>[57]</sup> have demonstrated that an age above 70 years is an independent risk factor for mortality in patients admitted with SAP. Based on these previous studies, the new severity criteria included CRP and age of the patient. In spite of these changes, the new severity criteria that employ routinely available data are simple and easy to remember.

Since the contrast-enhanced CT (CE-CT) is the mainstay of imaging patients with AP and recommended for the evaluation of the severity of AP<sup>[20,24-27,34,35,49]</sup>, the revised severity criteria included the CE-CT findings of the presence and extent of pancreatic necrosis, and the extent of peripancreatic inflammatory changes (Table 2). The revised Atlanta classification provided precise definitions of CE-CT findings, including peripancreatic necrosis, walled-off-necrosis and pseudocyst<sup>[21,22]</sup>. Although the revised Atlanta classification suggested that pancreatic necrosis can rarely be identified accurately during the first several days of hospitalization, CE-CT findings help us to decide special measures such as continuous regional arterial infusion (CRAI) of protease inhibitors and antibiotics, and continuous hemodiafiltration (CHDF)<sup>[58-60]</sup>. Once it is thought that contrast medium exacerbates pancreatitis<sup>[61-63]</sup>, but denied by another studies<sup>[64,65]</sup>. Since, however, there is a possibility that intravenous contrast media extend pancreatic necrosis and exacerbate renal impairment<sup>[61-63]</sup>, vigorous intravenous hydration for the purpose of intravascular resuscitation is important during and after CE-CT examination. Attending physicians must aware of the possibility that the contrast medium aggravates renal dysfunction associated with SAP.

The new severity criteria considered laboratory/clinical symptoms and radiographic features of CE-CT scans as independent risk factors. Indeed, Leung *et al.*<sup>[14]</sup> have demonstrated that CT severity index is a useful tool in assessing the severity and outcome of AP, and superior to Ranson score<sup>[10]</sup> and APACHE II scoring system<sup>[12-15]</sup> in predicting AP outcome. Thus, the CE-CT is not necessarily required to evaluate the severity of the patients with AP. Preliminary study revealed that the case-fatality

**Table 3 Verification of the revised severity criteria**

Total severity score (points)	Revised severity criteria	Criteria 2002
0	66	77
1	51	31
2	18	15
3	11 (1)	9
4	4	7
5	4 (2)	6
6	2 (1)	3 (1)
7	0	2 (1)
8	0	0
9	0	1
10	0	2 (2)
11	0	2
12	0	1
Total	156 (4)	156 (4)

Results shown are number of patients. Number in parenthesis indicates patients died of acute pancreatitis. The same patients with acute pancreatitis were evaluated by the revised criteria and by the criteria 2002. Total severity score of the revised criteria  $\geq 3$  points, while that of the criteria 2002  $\geq 2$  points was diagnosed as severe acute pancreatitis.

**Table 5 Relationship between the laboratory/clinical and the contrast-enhanced computed tomography severity scores**

Total CE-CT severity score (points)	Total laboratory/clinical severity score (points)							Total
	0	1	2	3	4	5	6	
0	0	0	0	0	0	0	0	0
1	56	40	13	5	1	0	0	115
2	3	5	2	1	0	2 (1)	1 (1)	14 (2)
3	2	1	1	3 (1)	3	2 (1)	1	13 (2)
Total	61	46	16	9 (1)	4	4 (2)	2 (1)	142 (4)

Results shown are number of patients. Number of patients died of acute pancreatitis is indicated in parenthesis. In these 142 patients, laboratory/clinical and contrast-enhanced computed tomography (CE-CT) examinations were evaluated at the same time.

in patients with the CE-CT severity score 1 was 3.3%, while that in those with severity score 2 and 3 points was 21.9% and 33.3%, respectively. Thus, the severity scores of CE-CT  $\geq 2$  points was defined as SAP (Table 2).

Analysis of case records of 1337 consecutive patients with AP in survey 2003<sup>[1,5,6]</sup> in that more than 5 items of 9 prognostic factors of the new severity criteria were recorded revealed that case-fatality rate of patients with severity score point 0 and point 1 was nearly the same (0.2% *vs* 0.7%), whereas that of patients with severity score 2 and 3 points was greatly different (2.6% *vs* 11.1%). Thus, the new criteria divided the severity of AP into mild (severity score  $\leq 2$  points) and SAP (severity score  $\geq 3$  points). Based on this classification, case-mortality rate of mild AP and SAP was 0.83% (9/1183) and 19.5% (30/154), respectively.

## VERIFICATION OF THE NEW SEVERITY CRITERIA

Usefulness of the new severity criteria was prospectively

**Table 4 Relationship between the revised laboratory/clinical or contrast-enhanced computed tomography severity score and incidence of organ failure**

Total severity score (points)	Incidence of organ failure	
	Laboratory/clinical	CE-CT
0	1.5%	0.0%
1	7.8%	4.3%
2	5.5%	42.9%
3	36.4%	46.2%
4	50.0%	-
5	75.0%	-
6	100.0%	-

Total number of patients evaluated by laboratory/clinical examinations was 156, whereas contrast-enhanced computed tomography (CE-CT) severity score was evaluated in 142 of these 156 patients at the same time.

studied in 156 patients with AP. CE-CT severity score was evaluated in 142 of these 156 patients at the same time with laboratory examinations. Overall case-mortality of 156 patients with AP was 2.6%, and was similar to that reported in nationwide survey in 2003<sup>[1,5,6]</sup>. Although some survey sheets had defect data of laboratory examinations, most frequently BE (defect value 41.0%) and PaO<sub>2</sub> (defect value 41.0%), these data were substituted by clinical signs of shock (defect value 0%) and respiratory failure (defect value 0%), respectively. Therefore, the severity score could be precisely calculated even if these laboratory data were defect values.

The revised severity criteria (Table 1) identified 13.5% of these 156 patients with AP as SAP, whereas 30.8% were diagnosed as SAP if the criteria 2002 were adopted. Case-mortality of SAP diagnosed by the revised criteria was 19.1%, whereas that by the criteria 2002 was only 8.3% due to large number of patients who are diagnosed as SAP (Table 3). The validity of the revised classification was further revealed by the incidence of complications of organ failure. Complications of organ failure were far greater in patients with SAP than in those with mild AP (Table 4). These results clearly indicate that the patients with SAP diagnosed by the revised criteria are suitable as applicants for the medical expense payment system<sup>[3]</sup>.

Since the new severity criteria consider laboratory data/clinical symptoms, and the CE-CT severity score as independent risk factors, SAP can be diagnosed either by the laboratory/clinical severity criteria or by the CE-CT severity criteria. There was no fatal case of mild AP diagnosed by the laboratory/clinical severity score regardless of CT severity score. Similarly, there was no fatal case of mild AP diagnosed by the CE-CT severity score regardless of laboratory/clinical severity scores (Table 5). Case-mortality rate of patients with SAP diagnosed by the laboratory/clinical severity score was 21.1%, whereas that in those diagnosed by the CE-CT severity score was 14.8%. Case fatality of SAP that fulfilled both laboratory/clinical (severity score  $\geq 3$  points) and CE-CT severity criteria (severity score  $\geq 2$  points) was as high as 30.8%. It is recommended, therefore, to perform CE-CT examination to clarify the prognosis in patients who were diagnosed as SAP by laboratory/clinical severity score. Because the

mortality rate of these patients with SAP was high, such patients should be transferred to advanced medical units with physicians specializing in intensive care, endoscopic treatment, radiological intervention, and biliary-pancreatic surgery<sup>[1,5,6]</sup>.

## CONCLUSION

The new severity criteria consist of laboratory examinations combined with clinical symptoms and the CE-CT severity score. The laboratory and/or clinical symptoms and the CE-CT findings are independent risk factors. SAP can be diagnosed either by the severity score alone, or by the CE-CT findings alone. Mortality rate of SAP that fulfilled both laboratory/clinical and CE-CT severity criteria was high. It is recommended, therefore, to perform CE-CT examination in those patients who were diagnosed as SAP by laboratory/clinical severity criteria. Patients with SAP who fulfill both severity criteria should be transferred to advanced medical units. The revised criteria are extremely useful to detect SAP at an early stage of AP.

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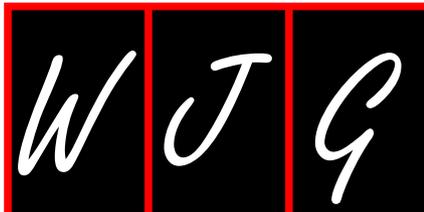
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## Achalasia: A review of clinical diagnosis, epidemiology, treatment and outcomes

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### Abstract

Achalasia is a neurodegenerative motility disorder of the oesophagus resulting in deranged oesophageal peristalsis and loss of lower oesophageal sphincter function. Historically, annual achalasia incidence rates were believed to be low, approximately 0.5-1.2 per 100000. More recent reports suggest that annual incidence rates have risen to 1.6 per 100000 in some populations. The aetiology of achalasia is still unclear but is likely to be multi-factorial. Suggested causes include environmental or viral exposures resulting in inflammation of the oesophageal myenteric plexus, which elicits an autoimmune response. Risk of achalasia may be elevated in a sub-group of genetically susceptible people. Improvement in the diagnosis of achalasia, through the introduction of high resolution manometry with pressure topography plotting, has resulted in the development of a novel classification system for achalasia. This classification system can evaluate patient prognosis and predict responsiveness to treatment. There is currently much debate over whether pneumatic dilatation is a superior method compared to the Heller's myotomy procedure in the treatment of achalasia. A recent com-

parative study found equal efficacy, suggesting that patient preference and local expertise should guide the choice. Although achalasia is a relatively rare condition, it carries a risk of complications, including aspiration pneumonia and oesophageal cancer. The risk of both squamous cell carcinoma and adenocarcinoma of the oesophagus is believed to be significantly increased in patients with achalasia, however the absolute excess risk is small. Therefore, it is currently unknown whether a surveillance programme in achalasia patients would be effective or cost-effective.

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**Key words:** Epidemiology; Achalasia; Incidence; Treatment; Oesophageal cancer risk

**Core tip:** Achalasia remains a disease of unknown aetiology. Multicentre studies could help elucidate the cause, especially as it presents with a similar phenotype to Chagas disease which is much better understood. Improved understanding of aetiology could guide novel treatments. Current treat choice in fit patients lies between pneumatic dilatation and laparoscopic Heller's myotomy. Botulinum toxin is appropriate and effective for those unfit for other intervention. Novel treatments such as metal stents and natural orifice surgery are being trialled.

O'Neill OM, Johnston BT, Coleman HG. Achalasia: A review of clinical diagnosis, epidemiology, treatment and outcomes. *World J Gastroenterol* 2013; 19(35): 5806-5812 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5806.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5806>

### INTRODUCTION

Achalasia is a motility disorder of the oesophagus, of unknown aetiology, which results in degeneration of the

myenteric nerve plexus of the oesophageal wall. The resultant abnormalities and diagnostic characteristics of achalasia include loss of oesophageal peristalsis and failure of relaxation of the lower oesophageal sphincter, particularly during swallowing<sup>[1]</sup>.

## DIAGNOSIS

Dysphagia is the cardinal symptom of achalasia. Diagnosis requires a high index of suspicion and exclusion of other causes. Diagnosis is confirmed by manometric, endoscopic and radiographic investigations. Oesophageal manometry is regarded as the gold standard in the diagnosis of achalasia, classically showing aperistalsis and failure of relaxation of the lower oesophageal sphincter<sup>[2]</sup>, as shown in Figure 1. Endoscopy is not accurate in the diagnosis of achalasia. However, it is still necessary to exclude a carcinoma at the lower end of the oesophagus<sup>[3]</sup>. Barium esophagogram can often show the pathognomonic “bird’s beak” appearance of the distal oesophagus with dilatation of the oesophagus proximally (Figure 2). However, this is often a finding in established disease and therefore a normal barium swallow does not rule out the diagnosis of achalasia. With the introduction of high resolution manometry, together with pressure topography, plotting the diagnosis of achalasia can now be classified into three subtypes; type 1 classic achalasia, type 2 achalasia with compression and pressurisation effects, and type 3 spastic achalasia<sup>[4]</sup>. This classification process can aid treatment decisions, with type 2 achalasia being the most responsive to pneumatic dilatation, Hellers myotomy and botulinum toxin and therefore having the best outcome<sup>[5]</sup>. Oesophageal emptying is determined by the distensibility of the oesophago-gastric junction. This can be assessed using an endoscopic functional luminal imaging probe (EndoFLIP). Recently, Dutch and American groups have demonstrated that this novel technique is a better predictor than lower oesophageal sphincter pressure for assessing response to treatment in achalasia, both symptomatically and when measured by gastric emptying by oesophageal emptying<sup>[6,7]</sup>.

## PATHOGENESIS

The pathogenesis of achalasia is not well understood but it is believed to be due to an inflammatory neurodegenerative insult with possible viral involvement. The measles and herpes viruses have been suggested as candidate viruses however molecular techniques have failed to confirm these claims and therefore the causative agent remains undiscovered<sup>[8]</sup>. Genetic and autoimmune components have also been suggested as origins of the neuronal damage however research to date has not found the exact cause<sup>[9]</sup>. Inflammatory changes within the oesophagus following the causative insult result in the loss of post-ganglionic inhibitory neurons in the myenteric plexus and a consequent reduction in the inhibitory transmitters, nitric oxide and vasoactive intestinal peptide. The excitatory

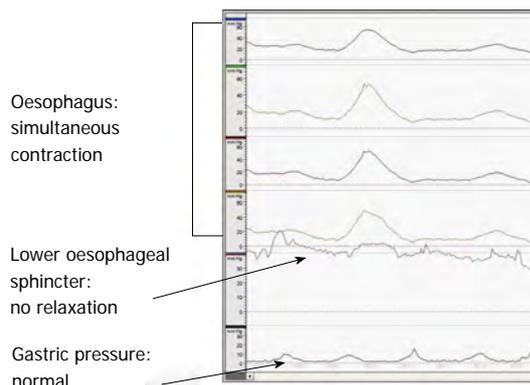


Figure 1 Oesophageal manometry demonstrating simultaneous contractions within the oesophagus and a non-relaxing lower oesophageal sphincter.

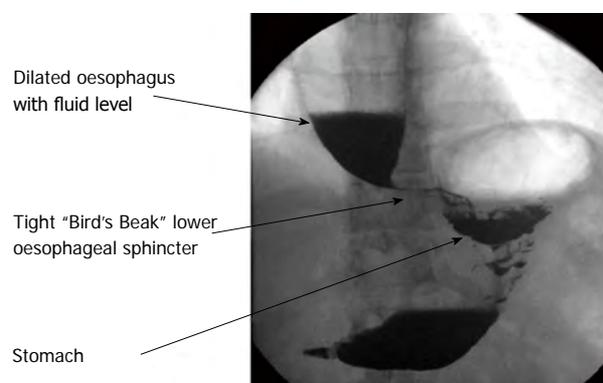


Figure 2 Barium swallow demonstrating typical “bird’s-beak” appearance of the lower oesophageal sphincter in achalasia. The oesophagus above this is dilated.

neurons remain unaffected, with the resulting imbalance between excitatory and inhibitory neurons preventing lower oesophageal sphincter relaxation<sup>[10]</sup>. Lack of peristalsis and a non-relaxing lower oesophageal sphincter cause progressive dysphagia. Regurgitation, particularly at night, with aspiration of undigested food and weight loss can be presenting features, particularly in established disease. Features which present in the early stages of the disease may be similar to that of gastro-oesophageal reflux, including retrosternal chest pain typically after eating and heartburn<sup>[11]</sup>. Due to initial non-specific symptoms in early stage disease and the low prevalence of achalasia worldwide, the condition often goes undiagnosed for many years, giving rise to features of late stage disease and their associated complications.

## EPIDEMIOLOGY

Achalasia is a relatively rare condition. A summary of studies published to date on achalasia incidence and prevalence is shown in Table 1<sup>[12-25]</sup>. The incidence of achalasia varied between studies, with reports as low as 0.03/100000 per year in Zimbabwe<sup>[22]</sup> to 1.63/100000 per year in Canada<sup>[14]</sup>. The majority of incidence rates re-

**Table 1** Summary of epidemiological studies of achalasia incidence and prevalence in adults

Study	Location	Years studied	Total number of achalasia patients	Prevalence rate (per 100000)	Incidence rate (per 100000/year)
Howard <i>et al</i> <sup>[12]</sup>	Edinburgh, Scotland	1986-1991	Not reported	Not reported	0.81
Birgisson <i>et al</i> <sup>[13]</sup>	Iceland	1952-2002	62	8.7	0.55
Sadowski <i>et al</i> <sup>[14]</sup>	Alberta, Canada	1995-2008	463	2.51 <sup>8</sup> 10.82 <sup>9</sup>	Not reported 1.63 <sup>9</sup>
Mayberry <i>et al</i> <sup>[15]</sup>	Great Britain and Ireland	1972-1983	6306	Not reported	Not reported
	Scotland		583	11.2	1.1-1.2 <sup>6</sup>
	Wales		197	7.1	Not reported
	Northern Ireland		153	9.8	Not reported
	Republic of Ireland		453	13.4	Not reported
Mayberry <i>et al</i> <sup>[16]</sup>	England		4920	10.8	0.9 <sup>7</sup>
Mayberry <i>et al</i> <sup>[16]</sup>	Cardiff, Wales	1926-1977	48	Not reported	0.4
Mayberry <i>et al</i> <sup>[17]</sup>	Nottingham, England	1966-1983	53	8.0	0.51
Arber <i>et al</i> <sup>[18]</sup>	Israel	1973-1983	162	7.9 <sup>1</sup>	0.8 <sup>5</sup>
				12.6 <sup>2</sup>	1.15 <sup>4</sup>
Earlam <i>et al</i> <sup>[19]</sup>	Rochester, United States	1925-1964	11	Not reported	0.6
Galen <i>et al</i> <sup>[20]</sup>	Virginia, United States	1975-1980	31	Not reported	0.6
Mayberry <i>et al</i> <sup>[21]</sup>	New Zealand	1980-1984	152	Not reported	1.0
Stein <i>et al</i> <sup>[22]</sup>	Zimbabwe	1974-1983	25	Not reported	0.03
Farrukh <i>et al</i> <sup>[23]</sup>	Leicester, England	1986-2005	14	Not reported	0.89 <sup>5</sup>
Ho <i>et al</i> <sup>[24]</sup>	Singapore	1989-1996	49	1.77	0.29
Gennaro <i>et al</i> <sup>[25]</sup>	Veneto, Italy	2001-2005	365	Not reported	1.59

<sup>1</sup>Rate in 1973 only; <sup>2</sup>Rate in 1983 only; <sup>3</sup>Rate between 1973-1978; <sup>4</sup>Rate between 1979-1983 only; <sup>5</sup>Rate only applicable for South Asian population of region; <sup>6</sup>Rate reported as 1.1 for men and 1.2 for women; <sup>7</sup>Rate only applicable to Oxford region of England; <sup>8</sup>Rate in 1996 only; <sup>9</sup>Rate in 2007 only.

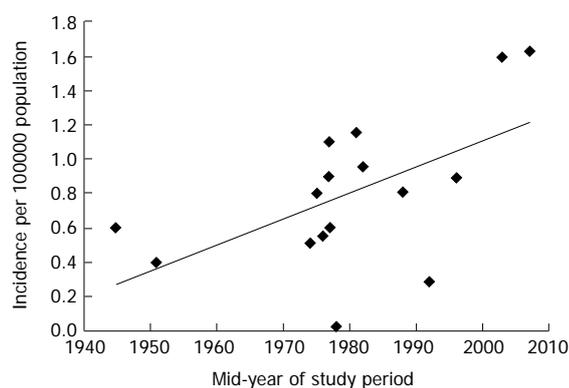


Figure 3 Achalasia incidence by mid-study time points.

ported clustered between 0.5-1.2 per 100000/year (Table 1). In an attempt to investigate changing incidence rates over time, we plotted incidence rates against the mid-timepoint within the study periods (Figure 3). As shown in Figure 3, incidence rates of achalasia appear to be rising, with most reports since the 1980s exceeding rates of 0.8/100000 per year, which has doubled to 1.6/100000 per year in post-2000 studies. Whether this reflects a true rise in incidence, or greater awareness and improved diagnosis of the condition remains uncertain though.

There are no distinct patterns of achalasia incidence in terms of age and sex distribution; it can affect both genders, all races and all ages<sup>[26]</sup>. A few studies have suggested a bimodal distribution of incidence by age, with peaks at around age 30 and 60 years<sup>[12,18,24]</sup>, while others have pointed towards a generally increased risk of achalasia with increased age<sup>[13,23,25]</sup>. Achalasia appears to affect males and females to largely equal extents<sup>[12,13,15,18,21,23-25,27]</sup>

although some investigations have detected slightly higher rates amongst females<sup>[16,19,28]</sup>. Only one study reported a higher achalasia incidence in men<sup>[14]</sup>. A study carried out by Mayberry *et al*<sup>[15]</sup> found achalasia to be significantly more common in the Republic of Ireland in comparison to its neighbouring countries (Table 1). Similarly, a study which reviewed the incidence of achalasia in New Zealand found differing incidence between ethnic groups<sup>[21]</sup>. The Pacific Islanders had an incidence of 1.3/100000 per year in comparison to those of Maori descent having an incidence of 0.2/100000 per year. This may reflect the influence of genetic factors in achalasia aetiology<sup>[21]</sup>.

A Canadian population-based study also considered the prevalence and survival rates of patients with achalasia<sup>[14]</sup>. Sadowski *et al*<sup>[14]</sup> found that the prevalence of achalasia rose from 2.51/100000 in 1996 to 10.82/100000 in 2007, despite a relatively stable incidence over the same time period (Table 1). The rise in prevalence was seen in both genders but was noted to be more pronounced in males, reflecting the fact that achalasia is a slowly progressive disease. This rise in prevalence was also evident in an Israeli study<sup>[18]</sup> and was noted in an Icelandic study between 1952 and 2002<sup>[13]</sup>. It is interesting to note that the Canadian study observed survival of achalasia patients to be significantly lower than the age-sex matched control population<sup>[14]</sup>. However, others have discerned that the majority of deaths in achalasia patients result from unrelated causes, leading to an equivalent life expectancy to the general population<sup>[29]</sup>.

## AETIOLOGY

There has been much debate over the aetiology of achalasia

lasia, with several potential triggers for the inflammatory destruction of inhibitory neurons in the oesophageal myenteric plexus being implicated. These include autoimmune responses, infectious agents and genetic factors.

### Auto-immune conditions

One recent study observed that patients with achalasia were 3.6 times more likely to suffer an autoimmune condition, compared with the general population<sup>[30]</sup>. Sjogren's syndrome, Systemic Lupus Erythematosus and uveitis were all significantly more prevalent in achalasia patients. The study also found the presence of a T-cell infiltrate and antibodies within the myenteric plexus of many patients with achalasia and an increased presence of human leukocyte antigen class II antigens<sup>[30]</sup>. Another study noted an overall higher prevalence of neural autoantibodies in patients with achalasia in comparison with a healthy control group<sup>[31]</sup>. Although no specific autoantibody was identified, this further supports the theory that achalasia has an autoimmune basis<sup>[31]</sup>.

### Infectious agents

The role of an infectious agent in the development of achalasia has been widely debated with several viral agents being implicated. For example, Chagas disease has a known infectious aetiology, and exhibits many similarities with achalasia<sup>[32]</sup>. In addition, there are several reports of varicella zoster virus and Guillain-Barre syndrome preceding the onset of achalasia<sup>[32]</sup>. Antibody studies have demonstrated increased titres to herpes and measles viruses in patients with achalasia in comparison to healthy control groups<sup>[33,34]</sup>. One study looking specifically at the link between the herpes simplex virus (HSV) and primary achalasia indicated the presence of HSV-1 reactive immune cells in the lower oesophageal sphincter of achalasia patients, suggesting that HSV-1 may be involved in the neuronal damage to the myenteric plexus leading to achalasia<sup>[35]</sup>. A further study of peripheral blood immune cells found that patients with achalasia showed an enhanced response to HSV-1 antigens<sup>[34]</sup>. In contrast, another investigation using PCR on myotomy specimens did not find any association between herpes, measles or human papilloma viruses and achalasia<sup>[36]</sup>. The current evidence for a causative infectious agent is contradictory and no clear causal relationship has yet been established.

### Genetic predisposition

The genetic basis for achalasia has not been widely investigated due to its low prevalence. One syndrome, known as the triple "A" syndrome, which consists of a triad of achalasia, alacrima and adrenocorticotrophic hormone resistant adrenal insufficiency is a known autosomal recessive disorder caused by gene mutations on chromosome 12. This syndrome, together with the prevalence of cases within children of consanguineous couples<sup>[37]</sup>, suggests the possibility for a genetic component to the aetiology of achalasia. There have been associations

with other genetic diseases including Parkinson's disease, Downs syndrome and MEN2B syndrome<sup>[10]</sup>. One recent study suggested the possibility of involvement of the rearranged during transfection gene, which is a major susceptibility gene for Hirschprung's disease (also linked with Down's syndrome)<sup>[9]</sup>. Mayberry *et al.*<sup>[38]</sup> conducted a study of first degree relatives of achalasia patients but concluded that inheritance was unlikely to be a significant causative factor due to the rarity of familial cases and exposure to common environmental and social factors within a family group may explain the presence of familial cases of achalasia.

It has been postulated that achalasia may incorporate a multi-factorial aetiology with an initiating event such as a viral or environmental insult resulting in oesophageal myenteric plexus inflammation. This inflammatory reaction may then initiate an autoimmune response in a susceptible group of genetically predisposed people, causing destruction of inhibitory neurons<sup>[39]</sup>.

## TREATMENT

The mainstay of treatment for achalasia is either pneumatic balloon dilatation or laparoscopic myotomy<sup>[40]</sup>. In pneumatic balloon dilatation, a balloon is positioned across the lower oesophageal sphincter and inflated, effectively rupturing the muscle of the affected segment. Surgical myotomy can be performed as either an open or laparoscopic procedure. The laparoscopic technique is now the most commonly performed. The procedure involves making a longitudinal division of the circular muscle of the lower oesophageal sphincter, extending this both proximally and distally onto the cardia<sup>[11]</sup>. Many surgeons advise the use of an anti-reflux procedure together with surgical myotomy, as these patients are at an increased risk of reflux following surgery<sup>[3]</sup>. A recent study compared partial anterior and partial posterior fundoplication following cardiomyotomy. It concluded that partial posterior fundoplication was superior to the anterior procedure due to significantly lower reintervention rates for postoperative dysphagia<sup>[41]</sup>.

The best comparative study between pneumatic dilatation and surgery to date has demonstrated remarkably similar outcomes in matched patients over a three year follow up period<sup>[42]</sup>. Therapeutic success at two years was noted in 86% of those treated by pneumatic dilatation and 90% of those who had laparoscopic Heller's myotomy. The regimen for pneumatic dilatation was rigorous with the option of multiple dilatations. The accompanying editorial suggests that choice should be determined by patient preference and local expertise<sup>[43]</sup>. A new endoscopic esophagomyotomy technique has been recently introduced: Peroral endoscopic myotomy involves dividing the inner circular muscle of the oesophagus. This requires sophisticated expertise and remains experimental<sup>[39]</sup>.

In patients for whom invasive procedures are not suitable, alternative treatment options may be considered including pharmacological intervention using long-acting

nitrates and calcium channel blockers. However, these are of limited benefit<sup>[44]</sup>. A further option is botulinum toxin injection into the lower oesophageal sphincter. This technique offers good short term results<sup>[45]</sup>. Most recently, metal stents have been used successfully<sup>[46]</sup>. Both of these options are generally only suited to those with several co-morbidities<sup>[9]</sup>.

## COMPLICATIONS

Patients with achalasia are at risk of developing the complications associated with disease progression as well as its treatment interventions. The complications of achalasia that can develop as a result of the natural course of the condition include aspiration-pneumonia<sup>[47]</sup>. Mega-oesophagus develops in 10% of inadequately treated cases and can ultimately require oesophagectomy<sup>[48]</sup>.

Squamous cell carcinoma is the most common oesophageal cancer in patients with achalasia and this is thought to result from the high level of nitrosamines produced by bacterial overgrowth due to stasis in the oesophagus leading to chronic inflammation and dysplasia<sup>[49]</sup>. There is considerable variation in the documented oesophageal cancer risk in achalasia. One review found that the prevalence of oesophageal cancer in achalasia was 3%, corresponding to a 50-fold increased risk<sup>[50]</sup>, while a prior review reported increased risks ranging from 0-33-fold greater than the general population<sup>[51]</sup>. Subsequent reports would also indicate a slightly more modest, but still significantly elevated, risk of oesophageal cancer 16-28-times greater than an age-sex matched control population<sup>[52,53]</sup>.

The risk of oesophageal adenocarcinoma is also increased in those with achalasia and may be a complication of longstanding reflux following successful interventional treatment<sup>[27,54]</sup>. A recent publication from The Netherlands demonstrated that 8.2% of 331 primary achalasia patients developed Barrett's oesophagus over a period of up to 25 years<sup>[55]</sup>. Two of these Barrett's cases progressed to develop oesophageal adenocarcinoma. Other studies have also reported elevated risks of Barrett's oesophagus and oesophageal adenocarcinoma in achalasia patients<sup>[27,56]</sup>.

A few studies have described even larger increased risks of oesophageal cancer amongst achalasia patients. One German study reported an risk of developing oesophageal cancer up to 140 times greater in patients with achalasia than the normal population<sup>[57]</sup>, which is equivalent to cancer risk in Barrett's oesophagus<sup>[58]</sup>. Furthermore, oesophageal cancer diagnosis in achalasia patients is often delayed, contributing to a poor mean survival after diagnosis of only 0.7 years<sup>[53,59]</sup>. These findings would support the need for endoscopic surveillance in achalasia patients.

However, despite the relative risk being increased, the absolute risk of cancer in patients with achalasia is still small and there would be a large number of examinations required to detect a single cancer. In fact it has been

estimated that for the detection of a single cancer there would need to be 681 endoscopic examinations undertaken<sup>[53]</sup>. Despite the potential complications associated with diagnosis, treatments and increased cancer risk, achalasia patients don't experience a significant compromise to overall life expectancy<sup>[29]</sup>. The most recent guidelines indicate that surveillance endoscopy is not indicated<sup>[60]</sup>.

## CONCLUSION

In conclusion, achalasia remains a relatively under-researched condition with many details on aetiology, true incidence, and risk of complications still unknown. There has been some progress over the past years into the aetiology of the condition but there is a need for further research to be carried out into this field to establish causative agents. Furthermore, clarification in relation to the need for an endoscopic screening program in patients with achalasia to detect the development of oesophageal cancer is required.

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## Molecular epidemiology and putative origin of hepatitis C virus in random volunteers from Argentina

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### Abstract

**AIM:** To study the subtype prevalence and the phylogenetic relatedness of hepatitis C virus (HCV) sequences obtained from the Argentine general population, a large cohort of individuals was analyzed.

**METHODS:** Healthy Argentinian volunteers ( $n = 6251$ ) from 12 provinces representing all geographical regions of the country were studied. All parents or legal guardians of individuals younger than 18 years provided informed written consent for participation. The corresponding written permission from all municipal authorities was obtained from each city or town where subjects were to be included. HCV RNA reverse transcription-polymerase chain reaction products were sequenced and phylogenetically analyzed. The 5' untranslated region (5'UTR) was used for RNA detection and initial genotype classification. The NS5B polymerase region, encompassing nt 8262-8610, was used for subtyping.

**RESULTS:** An unexpectedly low prevalence of HCV infection in the general population (0.32%) was observed. Our data contrasted with previous studies that reported rates ranging from 1.5% to 2.5%, mainly performed in selected populations of blood donors or vulnerable groups. The latter values are in keeping with the prevalence reported by the 2007 Argentinian HCV Consensus (approximately 2%). HCV subtypes were

distributed as follows: 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%). Two isolates ascribed either to genotype 1 (5%) or to genotype 3 (5%) by 5'UTR phylogenetic analysis could not be subtyped. Subtype 1a sequences comprised a highly homogeneous population and clustered with United States sequences. Genotype 1b sequences represented a heterogeneous population, suggesting that this genotype might have been introduced from different sources. Most subtype 2c sequences clustered close to the 2c reported from Italy and Southern France.

**CONCLUSION:** HCV has a low prevalence of 0.32% in the studied general population of Argentina. The pattern of HCV introduction and transmission in Argentina appears to be a consequence of multiple events and different for each subtype.

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**Key words:** Hepatitis C virus NS5B subtyping; Molecular epidemiology; Hepatitis C virus; Argentina; Hepatitis C virus 5' untranslated region

**Core tip:** This study reports an unexpectedly low prevalence of hepatitis C virus (HCV) (0.32%) in the general population of Argentina, with a subgenotype distribution of 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%) while two isolates ascribed either to genotype 1 (5%) or to genotype 3 (5%) could not be subtyped. Phylogenetic analysis of the NS5B region has enabled us to define the pattern of HCV introduction and transmission in Argentina as a consequence of multiple events that differed for each (sub)genotype studied. Furthermore, this report discusses the putative sources of HCV subgenotype introduction in Argentina.

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## INTRODUCTION

The analysis of extensive sets of sequences from hepatitis C virus (HCV) isolates throughout the world has revealed the existence of six major genetic groups or genotypes (named 1 to 6), and a large number of subtypes within these groups (designated a, b, c, *etc.*)<sup>[1]</sup>. Overall sequence divergence ranges from 31% to 33% among genotypes and from 20% to 25% among subtypes<sup>[1,2]</sup>; in a single patient, cloned E1/E2 sequences may differ by up to 10%. The reason for this great variation is a high mutation rate and high level of viral replication through an error prone RNA polymerase without proofreading

capacity. Consequently, in the infected individual, the virus circulates as a complex viral quasispecies<sup>[3]</sup> whose composition is subject to continuous changes due to competitive selection<sup>[4]</sup> and interactions among variants of with different levels of fitness<sup>[5]</sup>. The calculated average rate of fixation of mutations has consistently been found to range between 1.1 and  $1.5 \times 10^3$  mutations per site and per year<sup>[6,7]</sup>. The rate of fixation of mutations is not evenly distributed throughout the genome, which has highly variable regions within the envelope coding genes and well conserved regions, such as the 5' untranslated region (5'UTR). The practical consequence of the high conservation at 5'UTR for HCV genotyping is that this region contains insufficient variation to solve HCV classification at the level of viral subtypes<sup>[8]</sup>. Furthermore, genotype 6 variants other than 6a and 6b show 5'UTR sequences identical or similar to those of type 1 and, therefore, cannot be differentiated<sup>[9-11]</sup>. It has been reported that sequence analysis of the highly conserved region in NS5B known as the "Okamoto region" (nt 8282 to 8610 in the H77 reference genome)<sup>[8]</sup> provides the best concordance with the full length genome phylogeny for precise genotype identification. The same primers can amplify genotypes 1 to 5, and they are less efficient for genotype 6 isolates, but they facilitate analysis and classification of the amplified sequences<sup>[11]</sup>.

The prevalence of HCV infection in Argentina has been reported at 1.5% when all age groups are considered, and up to 2.0%-2.5% in adults<sup>[12]</sup>, a rate in keeping with the value reported by the Argentinian Consensus on Hepatitis C (approximately 2%) in 2007. A higher prevalence (4.9%-5.7%) has been described in small rural communities<sup>[13,14]</sup>. HCV genotype distribution in Argentina in groups at risk of HCV infection (*e.g.*, transfusion patients, hemodialysis patients, intravenous drug users, healthcare workers) has been reported at 53.5% for genotype 1, 23% for genotype 2 (mainly by subtype 2c [HCV-2c]), 8.6% for genotype 3, and 14.8% for mixed infections<sup>[13]</sup>. Similar results have been reported in studies on sequences deposited in GenBank (GB) and analyses by the Los Alamos database <http://hcv.lanl.gov>, with few genotype distribution changes (79.5% for genotype 1, 13.9% for genotype 2, 3.9% for genotype 3, 2.4% for genotype 4), but with some differences depending on the HCV subgroup at risk studied<sup>[16-18]</sup>. Phylogenetic characterization of genotype 4 isolates from Argentina has traced an independent origin of the three sequences studied<sup>[17]</sup>. Interestingly, HCV-2c was the most prevalent subtype (58%) in the city of Córdoba (Central geographical region of the country), followed by HCV-1b (33%), and to a lesser extent by HCV-1a (11%), HCV-3a (3%) and HCV-4a (3%)<sup>[19,20]</sup>.

Here, we report an unexpectedly low prevalence of HCV infection (0.32%) as measured by anti-HCV antibodies detected by using both a second generation enzyme immune assay (EIA) and a confirmatory immunoblotting, and HCV RNA detected by reverse transcription - nested polymerase chain reaction (RT-nested PCR)

**Table 1** Epidemiological profile of the population studied

Province/city of residence	Total number of individuals	Male/female number	Age <sup>1</sup> (yr), mean $\pm$ SE
Buenos Aires/C.A.B.A.	1461	685/776 <sup>a</sup>	33.4 $\pm$ 0.3 (11.2, 30) <sup>d</sup>
Catamarca	648	267/381	39.3 $\pm$ 0.5 (13.0, 38) <sup>d</sup>
Córdoba	1061	393/668 <sup>b</sup>	37.1 $\pm$ 0.4 (12.5, 35)
Chaco	353	175/178 <sup>b</sup>	40.2 $\pm$ 0.8 (14.1, 40) <sup>d</sup>
Chubut	172	83/89	37.4 $\pm$ 0.9 (12.1, 37)
Entre Ríos	474	225/249	38.5 $\pm$ 0.5 (11.6, 38)
Jujuy	176	105/71 <sup>b</sup>	35.2 $\pm$ 0.8 (10.3, 34) <sup>d</sup>
Río Negro	329	149/180	40.1 $\pm$ 0.7 (12.8, 39) <sup>d</sup>
Salta	561	230/331	41.3 $\pm$ 0.5 (12.8, 41) <sup>d</sup>
San Luis	195	52/143 <sup>b</sup>	41.6 $\pm$ 1.2 (16.3, 40) <sup>d</sup>
Santiago del Estero	375	164/211 <sup>b</sup>	38.0 $\pm$ 0.7 (13.2, 35)
Tucumán	446	209/237	37.7 $\pm$ 0.6 (12.0, 35)
Total	6251	2738/3513	37.5 $\pm$ 0.2 (12.7, 35)

<sup>1</sup>Data are expressed as mean  $\pm$  SE (SD, median). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , regarding the gender distribution (male/female ratio) within the whole population studied; <sup>d</sup> $P < 0.01$  regarding the mean age  $\pm$  SD from the whole population studied. C.A.B.A.: Ciudad Autónoma de Buenos Aires, the national capital city.

targetting the 5'UTR HCV RNA in a cohort of random Argentinian volunteers. The genotypes detected and the putative origin of the HCV sequences are discussed based on both their phylogenetic clustering and on such clustering relative to other Argentinian and worldwide derived sequences deposited in GB, in an attempt to trace how HCV could have been introduced in the local community here represented by the cohort studied.

## MATERIALS AND METHODS

Throughout the 2000-2007 period, a total of 6251 serum samples were collected from healthy volunteers from 12 Argentinian provinces, as well as from the Ciudad Autónoma de Buenos Aires (C.A.B.A. - the capital city of the country) as follows: Buenos Aires province and C.A.B.A.,  $n = 1461$ ; Catamarca,  $n = 648$ ; Córdoba,  $n = 1061$ ; Chaco,  $n = 353$ ; Chubut,  $n = 172$ ; Entre Ríos,  $n = 474$ ; Jujuy,  $n = 176$ ; Río Negro,  $n = 329$ ; Salta,  $n = 561$ ; San Luis,  $n = 195$ ; Santiago del Estero,  $n = 375$ ; and Tucumán,  $n = 446$  (Table 1).

Subjects included in this study [ $n = 6251$ ; 2738 men; mean  $\pm$  SE, 37.5  $\pm$  0.2 years; mean  $\pm$  SD = 37.5  $\pm$  12.7; median age = 35 years (range 10-70 years)] were recruited as volunteers from the general population, local schools, and police stations, after being informed about the aim of the survey. All parents or legal guardians of individuals younger than 18 years provided informed written consent for participation. The corresponding written permission from all municipal authorities was obtained from each city or town where subjects were to be included.

### Serological studies

The presence of anti-HCV antibodies was determined by using a second generation EIA test according to the manufacturer's recommendations (Abbott Diagnostics, North Chicago, IL, United States). Samples were further

analyzed with a second generation recombinant immunoblot assay (RIBA 2.0: Chiron Corporation, Emeryville, CA, United States).

### HCV-RNA detection and genotyping

Samples with serologically detectable anti-HCV antibodies were subjected to either RT-nested or RT-hemi-nested PCR amplification (see below). The 5'UTR region was used for RNA detection and initial genotype classification. The NS5B polymerase region, encompassing nt 8262-8610, was used for subtyping.

### RNA extraction

RNA was extracted from 140  $\mu$ L of serum by using the QIAamp Viral RNA Mini Kit (Qiagen Hilden, Germany). The measures to prevent contamination suggested by Kwok and Higuchi were strictly applied<sup>[21]</sup>.

### 5'UTR RT-nested PCR amplification and sequencing

The 5'UTR RT-nested PCR was performed as follows. RT was carried out for 45 min at 42 °C (GeneAmp 2700 PCR system, Applied Biosystems, Foster City, CA, United States), using 50 U M-MLV reverse transcriptase, RNase H Minus, Point Mutant (200 U/ $\mu$ L Promega, Madison, WI, United States), 20 U RNase inhibitor (40 U/ $\mu$ L Promega, Madison, WI, United States), 10 mmol/L of each dNTP (Roche, Basel, Switzerland), 20 pmols of antisense PCR primer NR5 5'TGCTCATGGTGCACGGTCTACGAG3' and 1  $\times$  buffer from the high fidelity *Pfu* turbo DNA polymerase (Stratagene, San Diego, CA, United States) in a final volume of 20  $\mu$ L. Then, 80  $\mu$ L of PCR mix containing 1  $\times$  *Pfu* turbo buffer, 20 pmol of sense primer NF5 5'GTGAGGAACTACTGTCTTCACG-CAG3' and 2.5 U *Pfu* turbo DNA polymerase were added to each tube. After an initial denaturation step of 2 min at 95 °C, 5 initial cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C were carried out, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 2 min at 72 °C, finishing with a single final step of 10 min at 72 °C. Five microliters of the product were used for nested PCR, by using internal primers the internal primers, K80 5'AGCGTCTAGCCATGGCGT3' and K78 5'CACTCG-CAAGCACCTATCAGGCAGT3'. The nested PCR mix consisted of 1  $\times$  *Pfu* turbo buffer, 10 mmol/L of each dNTP, 20 pmol of internal primers, and 2.5 U of *Pfu* turbo DNA polymerase in a final volume of 100  $\mu$ L. After a single denaturation step of 2 min at 95 °C, we carried out 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 2 min at 72 °C, and then, a final single step of 10 min at 72 °C. The amplified products of 240 nucleotides length were analyzed by electrophoresis onto 2% agarose gels stained with ethidium bromide. PCR products were purified by using the QIAquick PCR purification kit for direct sequencing on an Abi Prism 310 Genetic analyser (Applied Biosystems).

### NS5B RT-heminested-PCR amplification and sequencing

Extracted RNA was reverse transcribed using the de-

generate primer NS5B8704 5'GADGAGCADGAT-GTWATBAGCTC3' (nucleotide positions 8682-8704), where D = G + A + T, W = A + T and B = G + T + C, following the same conditions as for 5'UTR (see before). PCR was carried out by using the primer NS5B8256 5'TAYGAYACCMGNTGYTTTGACTC3' (nucleotide positions 8256-8278), where Y = C + T, M = A + C, and N = A + T + G + C, with an initial denaturation step of 2 min at 95 °C, five initial cycles of 30 s at 95 °C, 30 s at 43 °C, and 2 min at 72 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 46 °C and 2 min at 72 °C, and completed with a single final step of 10 min at 72 °C. Heminested PCR was performed with an initial denaturation step of 2 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 48 °C, and 2 min at 72 °C, completed with a single final step of 10 min at 72 °C using primers NS5B8256 and NS5B8641 5'GARTAYCTGGTCATAGCNTCCGT3' (nucleotide positions 8641-8619), where R = A + G, to obtain a final product of 386 nucleotides. Purification and sequencing were performed as mentioned above.

### Genotyping and subtyping-GB sequences

A GB query to Nucleotide collection (nr/nt), using the Megablast programme (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) was performed for each of the NS5B sequences obtained in this study. The 100 GB sequences with the highest sequence similarity to each of our samples were selected. A tree was constructed by using either the Neighbor-joining or Fast Minimum Evolution algorithm from a given matrix of distances using the Jukes-Cantor method to calculate the distances. For every query, we obtained a tree that situates each sequence with the most closely related reference from the GB. The most similar sequences were downloaded for further analysis, the genotype was assigned, and a putative origin of local isolates was inferred. GB was accessed to download already published sequences of Argentine origin by searching with the words "HCV" and "Argentina" on the website: <http://www.ncbi.nlm.nih.gov/sites/gquery>. Published sequences obtained by several authors<sup>[15,17,22,25]</sup> were downloaded in Fasta format, and their length was then adjusted using the GeneDoc program<sup>[24]</sup>.

### Phylogenetic analysis

Nucleotide sequences were resized by using GeneDoc and aligned by the CLUSTALW program<sup>[25]</sup>. Sequence similarity with other sequences from Argentina and with other GB sequences was ascertained by both distance and parsimony methods. Statistical support for each node in the trees drawn by both methods was obtained by performing 100 or 1000 bootstrap replicates of the original nucleotide sequence alignment. Phylogenetic analysis was carried out with the PHYLIP package<sup>[26]</sup>. Trees were drawn by using the Treeview program, v. 1.6.5<sup>[27]</sup>.

### Genetic divergence analysis

Genetic divergences were calculated by using the DNASP program (version 3.53)<sup>[28]</sup> in three populations:

our sequences grouped as a population, the closest GB sequences as another, and other Argentine sequences as the third one. Pairwise distances were calculated by using the MEGA3.1 program.

### Statistical analysis

They were performed by using either the GraphPath Prism (version 5.0 for Windows) or the SigmaStat software. The non-paired Student's *t* test was used to analyze the statistical differences between the mean age  $\pm$  SD of the whole population of the country regarding those values recorded from each Province (therefore, examining a data set from two groups), as well as for such comparison with previous studies. When such study was performed among three or more groups, the one-way analysis of variance (ANOVA) was applied. Pairwise distances were statistically compared by using the  $\chi^2$  test with Yates' correction (SigmaStat software). *P* values lower than 0.05 were considered statistically significant.

## RESULTS

### HCV prevalence in the general population of Argentina

A total of 6251 serum samples from a cohort of random volunteers was studied. Initially, 25 samples (0.40%) tested anti-HCV antibody positive as determined by EIA. However, 5 of the 25 samples failed to exhibit specific anti-HCV antibodies by immunoblotting analysis and also tested negative by RT-nested PCR to detect HCV RNA; hence, they were discarded for further studies.

HCV RNA was detected in serum samples from 7 out of 12 provinces. The prevalence of ongoing HCV infection as determined by RT-nested PCR from 12 of 23 provinces of Argentina, representing 73% of the country's total population, was 0.32%. The highest prevalence was detected in Buenos Aires province (0.62%; 9/1461), a geographical area inhabited by 40.52% of the total population of Argentina. In the remaining provinces studied, the prevalence ranged from 0% in Chaco, Chubut, Entre Ríos, Jujuy, and San Luis, to up to 0.53% in Santiago del Estero. Intermediate values were observed in Salta (0.18%) and Córdoba (0.19%), Río Negro (0.30%), as well as in Catamarca (0.46%) and Tucumán (0.45%).

Genotype 1 was the most prevalent, accounting for 55% (11/20) of infected individuals, 25% were subtype 1a (5/20) and 25% subtype 1b (5/20). The second in prevalence was genotype 2 accounting for 35% (7/20); most sequences were ascribed to subtype 2c (*n* = 5), except one that clustered much closer to subtype 2j reference sequences. Lastly, genotype 3 accounted for the remaining 10% (2/20; Table 2). One genotype 1, one genotype 2, as well as one genotype 3 isolates (according to their initial 5'UTR genotype assignment) were excluded from further analysis because of their respective NS5B RT-heminested PCR amplification failure.

### Phylogenetic analysis and genetic divergence

NS5B phylogenetic trees and divergence analysis were

**Table 2** Genotype and subtype assignment of the Argentinian isolates studied by using both 5'-untranslated region and NS5B sequences

HCV genotype	HCV[+] 5'UTR sequences	Relative	From total population (n = 6251)	HCV subtype	HCV[+] NS5B sequences	Relative	From total population (n = 6251)
1	11	55.00%	0.176%	1a	5	25.00%	0.080%
				1b	5	25.00%	0.080%
				1 <sup>1</sup>		5.00%	0.016%
2	7	35.00%	0.112%	2c	5	25.00%	0.080%
				2j	1	5.00%	0.016%
				2 <sup>1</sup>		5.00%	0.016%
3	2	10.00%	0.032%	3a	1	5.00%	0.016%
				3 <sup>1</sup>		5.00%	0.016%
				-	17	100.00%	0.320%
Total	20	100.00%	0.320%	-	17	100.00%	0.320%

<sup>1</sup>Untypeable at NS5B due to reverse transcription-heminested polymerase chain reaction amplification failure. HCV: Hepatitis C virus; 5'UTR: 5'-untranslated region.

**Table 3** Genetic divergence (DNASP software)

	Argentine general population sequences	GB deposited sequences from Argentina	Closest GB deposited sequences
HCV-1a			
Argentine general population sequences	0.034		
GB deposited sequences from Argentina	0.042	0.040	
Closest GB deposited sequences	0.034	0.037	0.032
HCV-1b			
Argentine general population sequences	0.082		
GB deposited sequences from Argentina	0.074	0.046	
Closest GB deposited sequences	0.054	0.050	0.045
HCV-2c			
Argentine general population sequences	0.087		
GenBank deposited sequences from Argentina	0.092	0.098	
Closest GB deposited sequences	0.076	0.080	0.076

Hepatitis C virus (HCV)-1b (Argentine general population sequences): 0.082 *vs* 0.054,  $P < 0.001$ ; 0.082 *vs* 0.074,  $P > 0.05$ ; HCV-1b [GenBank (GB) deposited sequences from Argentina]: 0.046 *vs* 0.050,  $P < 0.001$ ; HCV-2c (Argentine general population sequences): 0.087 *vs* 0.076,  $P < 0.001$ ; 0.087 *vs* 0.092,  $P > 0.05$ ; HCV-2c (GB deposited sequences from Argentina): 0.098 *vs* 0.08,  $P < 0.001$ . All  $P$  (HCV-1a)  $> 0.05$ . Genetic divergence (DNASP software), considering three separate HCV groups: our Argentine general population (sequenced in this study), Argentinian sequences already deposited in GB, and the closest GB worldwide sequences. All values obtained were statistically paired and compared by using the  $\chi^2$  test with Yates' correction.

performed by using the five HCV-1a, five HCV-1b, and five HCV-2c sequences. However, divergence could not be ruled out with those genotypes encompassing one single representative sequence (as occurred with 2j and 3a).

As expected, local NS5B sequences clustered together with their corresponding GB references (Figure

1), but the profile differed depending on the genotype. All of our HCV-1a Argentinian sequences clustered together with strong bootstrap support (92%; Figure 2A) and exhibited a low genetic divergence (0.034; Table 3). Furthermore, genetic divergence was not statistically different in comparison with other Argentinian sequences deposited in GB or with the closest non-Argentinian GB deposited sequences included in the study, suggesting a putative common source of infection/transmission.

In the case of Argentinian HCV-1b, we observed two clusters with very low bootstrapping support (19%-25%), and one sequence distantly located regarding such clusters (Figure 2B). Genetic divergence was higher (0.082) than that observed among GB deposited Argentinian 1b sequences but the differences were not statistically significant. Similarly, HCV-2c sequences were intermingled along the tree with no particular clustering (Figure 2C) and showed a high genetic divergence (0.087). Phylogenetic analysis of Argentinian HCV-1b and HCV-2c sequences suggested a different origin of infection/transmission when compared with HCV-1a sequences.

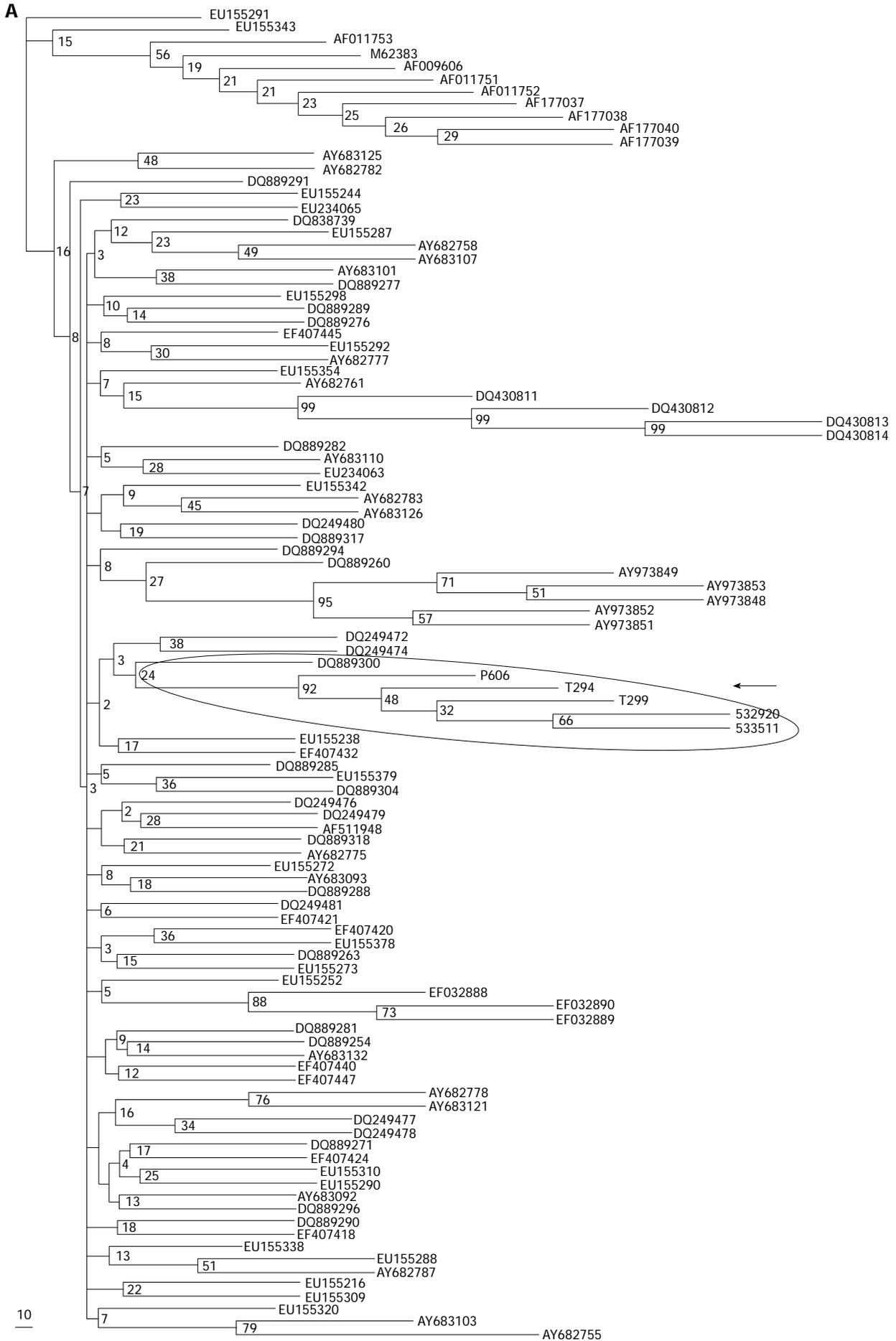
### Origin of closest GB sequences

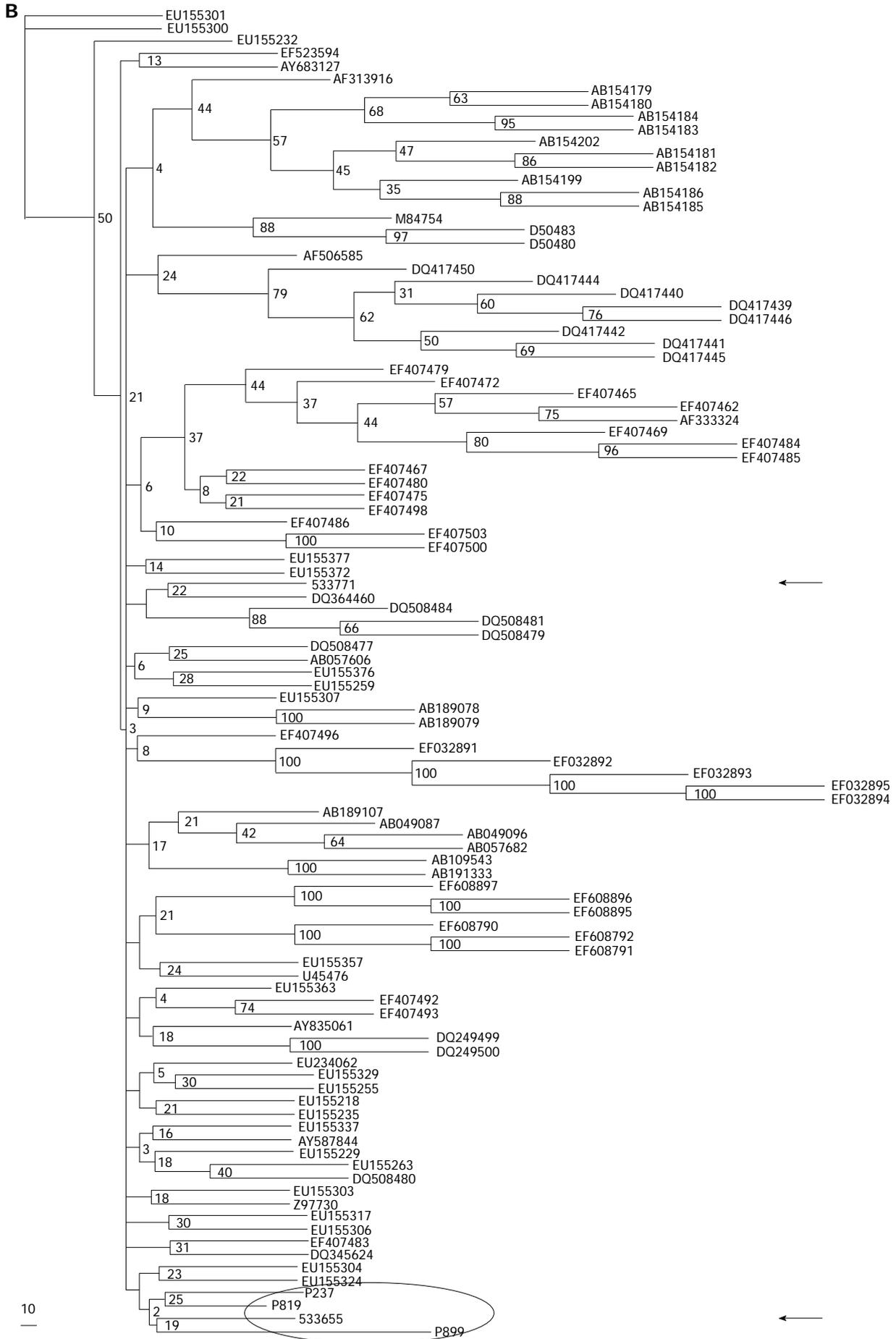
The closest GB sequences obtained by distance (UPGMA and NJ) and parsimony methods (DNAPARS) are represented in Table 4 (trees not shown). The geographic localization of the closest GB sequences is represented in Figure 3.

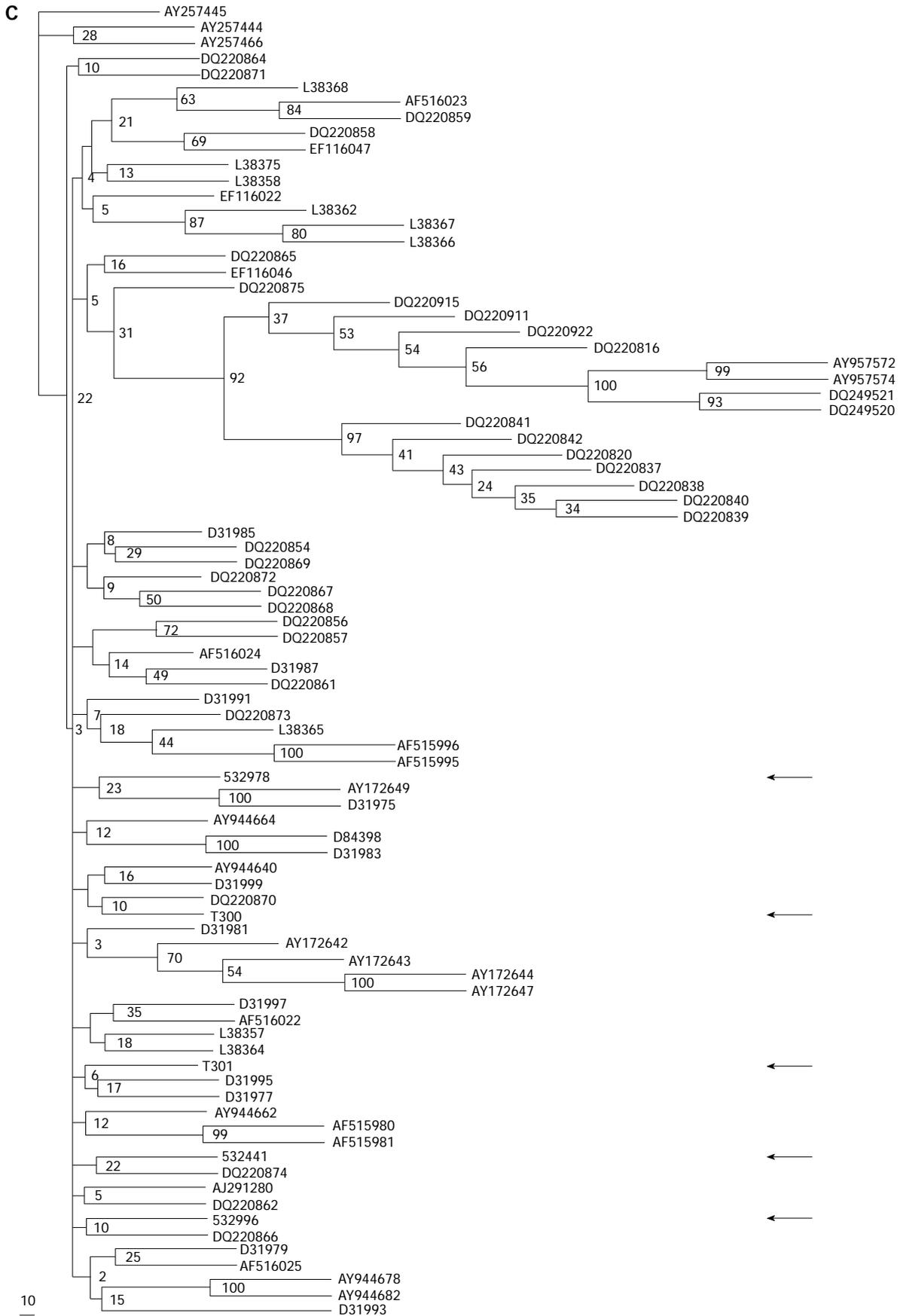
All local HCV-1a sequences grouped with GB United States sequences (St. Louis, Boston, or New York areas). The results suggest a narrow source of infection and not a multifocal event, and are consistent with the low degree of divergence found in the Argentine general population, despite the fact that the subjects studied resided in three different provinces (Buenos Aires, Córdoba, and Río Negro), and from whom serum sampling was performed, several hundred of kilometers apart from each other.

HCV-1b sequences grouped with those from all over the world, including Europe (Spain, Russia), Asia (China), Africa (Madagascar, Tunisia) and North America (United States), and represented a heterogeneous population (Table 3).









**Figure 2** Phylogenetic trees performed by the Neighbor-Joining method. NS5B sequences obtained from the volunteers whose respective hepatitis C virus (HCV) isolates had been classified as HCV-1a ( $n = 5$ ), HCV-1b ( $n = 5$ ) or HCV-2c ( $n = 5$ ) were respectively analyzed in panels A, B and C together with 100 HCV-1a, 100 HCV-1b or 81 HCV-2c sequences downloaded from the GenBank. The respective consensus trees are shown after analyzing 100 replicate trees for each HCV subtype. A distance scale (in nucleotide substitutions per position) is shown in each panel. Arrows indicate sequences obtained from Argentinian volunteers.

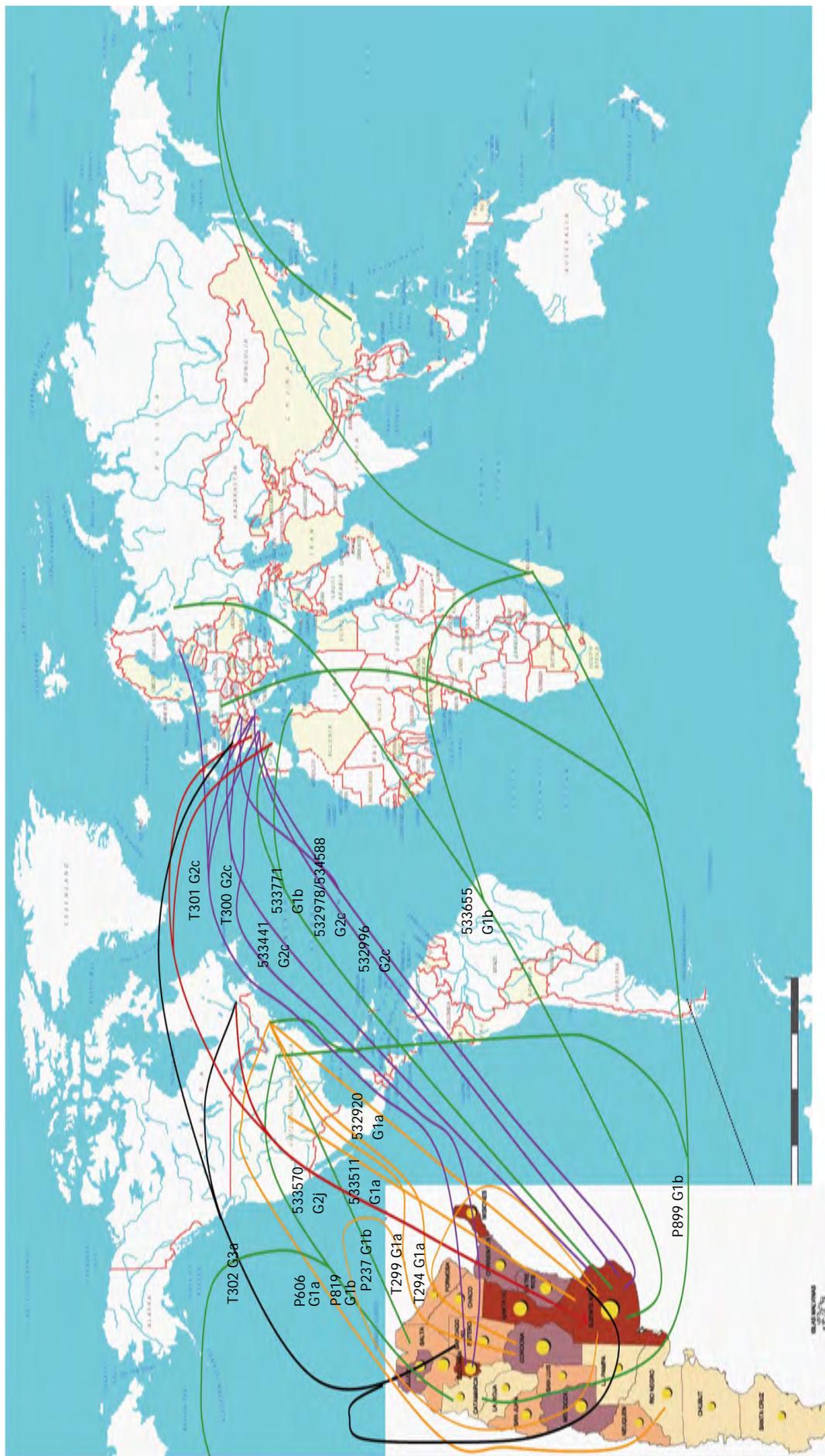


Figure 3 Each line links the Argentinian location of the sequence named on the top of the line with the world location/s of the most closely related sequence/s published in GenBank.

**Table 4** Subtype assignment of each hepatitis C virus Argentinian sequence obtained in this study

Isolate	NS5B genotype	Closest GB deposited sequences	Origin
532920	1a	DQ8893001bos	United States (Massachusetts, Boston area)
533511	1a	EU155310usa EU155290usa AY683093usa	United States
P606	1a	DQ8893001usa AY682755alb AY683103alb AY682775alb	United States (Albany, NY)
		DQ889318bos	United States (Massachusetts, Boston area)
T294	1a	DQ889300bos DQ313467arg DQ313464arg DQ313465arg DQ313466arg	Argentina
		DQ889300bos	United States (Massachusetts, Boston area)
T299	1a	DQ889300bos DQ889296bos AY172640arg	United States (Massachusetts, Boston area) United States (Massachusetts, Boston area) Argentina
		DQ889296bos	United States (Massachusetts, Boston area)
533655	1b	AF506602rus	Russia (Western Siberia)
		DQ345627mad	Madagascar (Antananarivo)
533771	1b	DQ508484tun DQ508481tun DQ508479tun	Tunisia (Tunis)
		EF608896bcn	Spain (Barcelona)
P237	1b	EU155224ten EU155304ten	United States (Tennessee)
P819	1b	AY835061chn DQ249500usa DQ249499usa	China (Foshan) United States (factor VIII concentrate)
		DQ345626mad	Madagascar (Antananarivo)
P899	1b	DQ345626mad AJ132997ger AY682461alb AY683105alb	Madagascar (Antananarivo) Germany United States (Albany, NY)
		DQ249501usa	United States (factor VIII concentrate)
532441	2c	L38363swi D31981ita	Switzerland Italy (France from Italians)
532996	2c	L38365swi AF15995mar AF515996mar	Switzerland France (Marseille)
		DQ220866tou D31975ita	France (Toulouse) Italy (France from Italians)
532978	2c	AY172649ita	Italy (France from Italians)
534588		D50409(BEBE1)ita	
T300	2c	DQ220870fr	France (Toulouse)
T301	2c	AF516025mar EF195026tall AY944641gen	France (Marseille) Estonia (Tallinn) Italy (Genoa)
		D31977ita	Italy (France from Italians)
533570	2j	AY89526que AY894529que AY894550que EF116050que	Canada (Quebec)
		DQ220919tou DQ220918tou	France (Toulouse)
		D86530bcn	Spain (Barcelona)
T302	3a	EF116078que AJ291256ssd AJ867113arg AJ867159arg AJ867116arg AJ867115arg AJ867114arg	Canada (Quebec) France (Seine Saint Denis district) Argentina

The 3<sup>rd</sup> column from the left shows the GenBank (GB) sequences that are most similar to each of our samples, and the 4<sup>th</sup> column from the left exhibits the origin of the GB already deposited sequences.

HCV-2c sequences from the Argentine general population formed a heterogeneous group with a completely different pattern as compared with HCV-1b. The sequences clustered with GB sequences originated from HCV patients in Italy. Even the sequences reported from Southeastern France were obtained from Italians living in this region. Only one of the clustered GB sequences was documented in Estonia. Three of our 2c sequences were detected in Buenos Aires/C.A.B.A. and two in Tucumán (approximately 1200 km from C.A.B.A.).

When blasted with GB sequences, the single sequence assigned to HCV-2j clustered with those from Canada, France, and Spain, showing the high heterogeneity among the HCV-2j sequences analyzed.

Regarding the single HCV-3a local sequence, similarities were found with one sequence from Canada and another one from France, and with an additional group of five sequences reported in Argentina.

## DISCUSSION

Here we report a very low prevalence of HCV infection (0.32%) in a large cohort of random volunteers from Argentina, contrasting with the 2% prevalence previously reported in studies based on selected populations in small communities, or even higher rates among highly vulnerable groups<sup>[12,29]</sup> (Hepatitis C Argentinian Consensus, 2007). The observed prevalence is lower than that reported in neighboring countries, such as Brazil (1.5%), Uruguay (1%), and Chile (0.85%)<sup>[12]</sup>. The highest prevalence was detected in Buenos Aires province and C.A.B.A., both making up the region that received the greatest number of European immigrants, especially during the first half of the 20<sup>th</sup> century (70.38% of all immigrants, 20.8% residing in C.A.B.A.; <http://www.mininterior.gov.ar/poblacion/estadisticas.asp> Censo2001). In this regard, recent data shows that at present most of the European immigrants from Italy and Spain are over 60-year-old ([http://www.mininterior.gov.ar/provincias/archivos\\_prv25/6-%0Perfil\\_Migratorio\\_de\\_la\\_Argentina.pdf](http://www.mininterior.gov.ar/provincias/archivos_prv25/6-%0Perfil_Migratorio_de_la_Argentina.pdf)).

The HCV isolates studied here did not form a close national cluster separate from the GB sequences. Interestingly, genetic divergence and phylogenetic analyses showed a different profile depending on the subtype analyzed. In this sense, the HCV-1a samples, detected from subjects residing in distantly placed cities/towns (hundreds of kilometers apart from each other) from three provinces, made up a highly homogeneous population, whereas the HCV-1b and HCV-2c samples were more heterogeneous, suggesting a different profile of epidemiological origin/transmission of infection for each subtype. The high homogeneity of subtype 1a and its similarity with sequences reported from United States suggest that HCV-1a was introduced in Argentina by a common infectious source from this geographic area. This finding agrees with the model of recent HCV genotype diversification in Central and South America<sup>[30-32]</sup>

compared with other continents. HCV-1b isolates formed separate clusters that were most similar to European sequences, suggesting multiple focal transmission events, likely with independent geographical origins. Interestingly, the subject whose HCV isolate showed an HCV-1b phylogenetic relationship with a Russian HCV-1b sequence stated such ethnicity. HCV-2c represents an important contribution to Argentinian HCV epidemiology (at least, 25% in this study), supporting previous observations (23%)<sup>[15]</sup>. Most of the 2c isolates clustered close to sequences reported from Italy and Southern France. In general, the 2c sequences deposited in GB represent a highly heterogeneous population with huge genetic diversity in Ghana, Guinea, Benin, and Burkina Faso in Africa<sup>[33]</sup>, suggesting that HCV-2c has long been present in human populations, especially in these parts of Africa, and that it spread to Egypt, Europe and elsewhere in the 20<sup>th</sup> century<sup>[34]</sup>. It has been proposed that the introduction of HCV-2c in Italy was a consequence of close contacts between native Africans and soldiers and colonials during the colonial wars in 1882-1896 and 1911-1912<sup>[35,36]</sup>. A high prevalence of HCV-2c was observed among individuals in Italy<sup>[37-40]</sup> and Southern France, all related with Italian immigrants<sup>[41]</sup>. Coincidentally, a substantial percentage of the Buenos Aires population descends from Italian immigrants that arrived in the 20<sup>th</sup> century. Taken together, our results suggest that the introduction of HCV-2c in Argentina may have been the result of a multiple event, likely related to waves of Italian immigration. In this regard, it is worth mentioning that a high prevalence (approximately 50%) of this genotype has been reported among chronic HCV patients from Córdoba province<sup>[19,20,29]</sup>, as compared with data from Buenos Aires and C.A.B.A. patients<sup>[15]</sup> and even higher rates (90%) from patients residing in Cruz del Eje, a small rural town located in the Northern region of Córdoba province, where HCV prevalence was reported to be 5%<sup>[29]</sup>. In contrast, the present study could not detect the circulation of such genotype from the general population studied in the city of Córdoba (encompassing the whole group from the homonymous province). Several hypothetical factors might have contributed to the observed discrepancy, among them, it seems worth mentioning: (1) the previously reported overall low prevalence of infection in the city of Córdoba<sup>[29]</sup> and in this study; (2) the lower contribution of HCV-2c to the total HCV genotype prevalence in such capital city located in the central region of the province, as compared with Cruz del Eje<sup>[20,29]</sup>; (3) the dissimilar nature of studied groups (patients versus general population), hence showing a dissimilar HCV infection prevalence, and consequently having lower probability to pick up HCV positive samples; and (4) the mean age  $\pm$  SE of all the analyzed populations ( $49.77 \pm 2.15$  for patients from Córdoba and other locations of Córdoba Province ( $n = 26$ )<sup>[29]</sup>,  $66.15 \pm 1.52$  years for patients from Cruz del Eje ( $n = 49$ )<sup>[29]</sup>, as compared with  $37.1 \pm 0.4$  in this study (SD = 12.5; median age = 35 years;  $n = 668$ ). However, the last mentioned factor failed to reach statis-

tical significance when the one way analysis of variance was carried out ( $P = 0.1177$ ).

The recorded HCV-3a sequence exhibited similarities with isolates from France and Canada and other Argentinian isolates, in concordance with a more recent, worldwide expansion of this subtype<sup>[22]</sup>. The HCV-2j sequence showed similarities with French, Canadian, and Spanish HCV sequences. No other genotypes (4, 5 or 6) were detected in the Argentine general population studied.

In conclusion, NS5B analysis allowed an accurate classification of subtypes and enabled to perform the study of the evolution and origin of HCV infection. Here, we report a very low prevalence of HCV in the Argentine general population (0.32%). Phylogenetic analysis suggests diverse profiles of epidemiological expansion of HCV in Argentina: HCV-1a might have occurred from a putative common source, whereas HCV-1b and HCV-2c might have been introduced into the country following fluxes of immigration from other endemic areas, especially from Europe. The significantly high number of HCV-2c sequences compared to the reported data from neighboring countries may be the consequence of the high percentage of Italians migrating to Argentina from an area where such subtype was (and still is) highly prevalent. Argentina is a good example of how human practices, together with global expansion and human migration flows, have increased the HCV spread over the world. Adherence to standard universal precautions to avoid transmission should be strictly followed even in countries with a low prevalence of HCV.

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## COMMENTS

### Background

Hepatitis C virus (HCV) is a leading cause of chronic liver disease. HCV is distributed globally, affecting all countries with an estimated worldwide prevalence of 2.3% (approximately 160 million people) of the whole general population. Comparisons of HCV nucleotide sequences derived from individuals from different geographical regions revealed the circulation of at least six major HCV genotypes and more than 50 subtypes. Accurate HCV genotyping in chronically infected patients is crucial not only for epidemiological studies but also from a clinical standpoint, since the HCV genotype orientates the treatment strategy.

### Research frontiers

Direct sequencing, also referred as "population" sequencing, is the gold standard for HCV genomic sequence analysis. The viral genome region(s) sequenced must be carefully chosen, because not all of them provide accurate typing and subtyping. Since genotyping methods based on the exclusive analysis of the 5'NCR may lead to up to 10% mistyped results, there is a need to extend the analysis to coding regions such as NS5B or core. In this regard, the knowledge of the implicated HCV genotype in each patient contributes to select the appropriate treatment. Those infected with HCV genotype 1 must be treated with a triple combination of pegylated interferon- $\alpha$  (IFN- $\alpha$ ), ribavirin and either

telaprevir or boceprevir, whereas patients infected with other genotypes must still be treated with pegylated IFN- $\alpha$  and ribavirin alone. Moreover, HCV genotyping based on phylogenetic analysis, and - in case a representative sampling of a given (sub)genotype is obtained from an area - Monte Carlo Markov Chains Bayesian coalescent analysis may respectively lead to trace the origin and if such condition is met - the putative date of the Most Recent Common Ancestor of sequences.

### Innovations and breakthroughs

This is a molecular epidemiological study performed in a large cohort of the local general population from 12 out of 23 Argentine provinces, as well as from the Autonomous city of Buenos Aires (the national capital). Unexpectedly, it shows a low prevalence of HCV (about 0.32%) in a general population cohort which included 6251 individuals. This low prevalence suggests that HCV could have been "recently" introduced in Argentina, as proposed by coalescent studies performed in restricted local areas of this country by other authors, where a predominant (sub)genotype was found, allowing such analysis. HCV subtypes were distributed as follows: 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%). HCV-1a sequences comprised a highly homogeneous population and clustered with United States sequences. HCV-1b sequences represented a heterogeneous population, suggesting that this genotype might have been introduced from different sources. Most HCV-2c sequences clustered close to the 2c reported from Italy and Southern France. Phylogenetic analysis is used by the authors to trace the putative source of HCV transmission and suggests that introduction of local HCV in this country is a consequence of multiple events that differed for each subtype studied. Diverse epidemiological patterns of HCV spread in Argentina might have occurred.

### Applications

These new data could be useful to implement suitable measures regarding HCV surveillance by Argentine Public Health authorities.

### Terminology

HCV genotype: group of HCV variants assigned to a given genetic groups (1-6) which differs from others by 31%-33% at the nucleotide level. HCV subtype (sub-genotype): group of more closely related HCV variants assigned to a given genetic group which differs from others by 20%-25% at the nucleotide level (named with lower case letters: *i.e.*, a, b, c, *etc.*).

### Peer review

This is a very well done and written molecular epidemiological study which considers the investigation of the prevalence of HCV infection and subtype frequencies among adults in Argentina. It should be underlined that authors have investigated a large amount of general population from 12 provinces representing all the geographical regions of the country.

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## Aberrant TGF- $\beta$ 1 signaling contributes to the development of primary biliary cirrhosis in murine model

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### Abstract

**AIM:** To investigate whether transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) signaling pathway is involved in the pathogenesis of primary biliary cirrhosis (PBC).

**METHODS:** A murine model of PBC was developed by injection of polyinosinic polycytidylic acids (poly I : C) in C57BL/6 mice, and the liver expressions of TGF  $\beta$ 1, TGF- $\beta$  receptor I (T $\beta$ R I), TGF- $\beta$  receptor II (T $\beta$ R II), p-Smad2/3, monoclonal  $\alpha$ -smooth muscle actin antibody ( $\alpha$ -SMA) and  $\alpha$ 1 (I) collagen in the mouse model and control mice were evaluated by immunohistochemistry, immunoblotting and real-time polymerase chain reaction (RT-PCR). Lymphocyte subsets in liver were analyzed using flow cytometry.

**RESULTS:** The mouse model had several key phenotypic features of human PBC, including elevated levels of alkaline phosphatase, antimitochondrial antibodies, portal bile ducts inflammation, and progressive collagen deposition. Compared with control mice, protein and mRNA levels of TGF  $\beta$ 1, T $\beta$ R I, T $\beta$ R II, p-Smad2/3,  $\alpha$ -SMA and  $\alpha$ 1 (I) collagen in liver ( $1.7 \pm 0.4$  vs  $8.9 \pm 1.8$ ,  $0.8 \pm 0.2$  vs  $5.1 \pm 1.5$ ,  $0.6 \pm 0.01$  vs  $5.1 \pm 0.1$ ,  $0.6 \pm 0.3$  vs  $2.0 \pm 0.3$ ,  $0.9 \pm 0.4$  vs  $3.4 \pm 0.6$ ,  $0.8 \pm 0.4$  vs  $1.7 \pm 0.3$ ,  $1.1 \pm 1.2$  vs  $11.8 \pm 0.6$ ,  $P < 0.05$ ), and the total number and percentage of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> and CD8<sup>+</sup> lymphocytes ( $0.01 \pm 0.001$  vs  $0.004 \pm 0.00$ ,  $0.12 \pm 0.04$  vs  $0.52 \pm 0.23$ ,  $P < 0.01$ ) were higher in the mouse model.

**CONCLUSION:** TGF $\beta$ 1 might play a dual role in the development of PBC: it suppresses inflammatory response but operates to enhance fibrogenesis. The aberrant activity of TGF- $\beta$ 1 signaling contributes to the development of PBC.

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**Key words:** Primary biliary cirrhosis; Transforming growth factor- $\beta$ 1; Regulatory T cell; Liver

**Core tip:** Primary biliary cirrhosis (PBC) is an autoimmune liver disease. Recent studies suggest that transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) signaling pathway might play an important role in the pathogenesis of PBC. However, whether TGF- $\beta$ 1 signaling pathway is involved in the development of PBC is still unknown. The studies have provided new data of TGF- $\beta$ 1 signaling pathway involving the pathogenesis of PBC, which will pose significant impact on our understanding of the pathogenesis of PBC. TGF- $\beta$ 1 signaling pathway is a potential target for PBC treatment.

Liu B, Zhang X, Zhang FC, Zong JB, Zhang W, Zhao Y. Aberrant TGF- $\beta$ 1 signaling contributes to the development of primary

biliary cirrhosis in murine model. *World J Gastroenterol* 2013; 19(35): 5828-5836 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5828.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5828>

## INTRODUCTION

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts. Damage of bile ducts is associated with cholestasis, and eventually leads to liver failure<sup>[1]</sup>.

Cytokines are involved in cell-to-cell interaction *via* specific receptors, inflammatory response amplification, immune regulation and fibrogenesis. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a prominent antiproliferative and profibrogenic cytokine that signals through TGF- $\beta$  receptor II (T $\beta$ R II), and receptor I (T $\beta$ R I), that in turn phosphorylate Smads at the mad homology 2 domain<sup>[2]</sup>. Perturbation of TGF- $\beta$ 1 signaling has been implicated in several developmental disorders and in various human diseases including cancer, fibrosis and autoimmune disease<sup>[3-5]</sup>. Mice transgenic of a dominant negative form of T $\beta$ R II, under the CD4 promoter lacking the CD8 silencer<sup>[6]</sup>, spontaneously developed features characteristic of PBC<sup>[7]</sup>. A compromised cytoarchitecture and polarized trafficking of TGF- $\beta$ 1 signaling molecules including embryonic liver fodrin and Smad3 were also noted in the pathogenesis of PBC<sup>[8]</sup>. Moreover, TGF- $\beta$ 1 was a marker for fibrosis and reflected severity of disease in patients with PBC<sup>[9,10]</sup>. Therefore, aberrant TGF- $\beta$ 1 signaling contributes to a loss of self tolerance to autoantigens in the liver, which in turn leads to autoimmunity.

We developed an animal model of PBC by polyinosinic polycytidylic acids (poly I : C) injection in genetically susceptible C57BL/6 female mice that would allow the analysis of the early cellular events of PBC<sup>[11,12]</sup>. We found that TGF $\beta$ 1 played a dual role in the development of PBC: it suppressed inflammatory response but operated to enhance fibrogenesis. The aberrant TGF- $\beta$ 1 signaling contributed to the development of PBC.

## MATERIALS AND METHODS

### PBC animal model

Adult 6-8 wk-old C57BL/6J (H-2b) mice were purchased from Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC, Beijing, China). They were maintained separately at the Department of Laboratory Animal, Peking Union Medical College Hospital (PUMCH), China, under controlled conditions (22 °C, 55% humidity, and 12 h day/night). All animals received adequate care according to good laboratory practice guidelines. The study protocol was approved by Committee of Animal Experimentation, PUMCH and CAMS. Female C57BL/6 mice were injected with 5 mg/kg

poly I : C (Invivogen Co. San Diego, United States) or normal saline (NS) as controls twice a week for 24 consecutive weeks, according to the protocol of Okada<sup>[11]</sup>.

At weeks 8 and 24, six mice of each group were sacrificed by cervical dislocation. Livers were fixed in buffered formalin (10%). Sera and tissue specimens were stored at -80 °C. The serum levels of alkaline phosphatase (ALP) and alanine amino-transferase (ALT) were measured by commercially available kit (WAKO Pure Chemical Industry, Osaka, Japan) exactly according to the manufacturer's protocol.

### Antimitochondrial antibodies detection

Antimitochondrial antibodies (AMA) and M2 were detected by the commercial immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) kits (EU-ROIMMUN, Germany) and immunoblotting kits (IMTEC Corporation, Germany), according to the manufacturer's protocol. Fluorescein isothiocyanate (FITC) or horseradish peroxidase (HRP)-conjugated monoclonal goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) was used as the secondary antibody. Plates were read at 450 nm with a microplate reader (Bio-RAD Model 550, Tokyo, Japan). Sera with optical density (OD) values greater than the mean  $\pm$  2SD from the negative controls were regarded as AMA positive.

### Histopathology

Formalin-fixed, paraffin-embedded tissue sections were cut into 5  $\mu$ m slices for routine hematoxylin and eosin staining. Tissues were also stained with Azan to detect collagen deposition<sup>[13]</sup>. Briefly, sections were deparaffinized in xylene, dehydrated, rehydrated in distilled water, immersed in 5% potassium dichromate solution for 30 min, and stained with azocarmine G for 30 min. Sections were then immersed in 3% 12 tungsto (IV) phosphoric acid n-hydrate solution for 1 h and stained with aniline blue-orange G for 20 min.

### Immunohistochemistry

Antibodies against CD4 (1/200; L3T4, eBioscience) and CD8 (1/100; 53-6.7; Biolegend) were used for immunohistochemical staining of the portal tract infiltrates. Anticytokeratin 7 (1/50; RCK 105; BD Bioscience, San Jose, CA, United States) was used to detect biliary cell. Antibodies against TGF- $\beta$ 1 (1/200; V), T $\beta$ R I (1/200; T-19), T $\beta$ R II (1/200; C-16), p-Smad2/3 (1:50; Ser 433/435) (all obtained from Santa Cruz Biotechnology, Dallas, Texas, United States) and monoclonal  $\alpha$ -smooth muscle actin antibody ( $\alpha$ -SMA, 1:250; 1-4A; Sigma, St. Louis, MO, United States) were used to detect the expressions of TGF- $\beta$ 1 signaling proteins. Briefly, after deparaffinization, sections were incubated in a Decloaking Chamber (Biocare Medical, CA, United States) set point: SP1 123 °C for 2 min, SP2 85 °C for 10 s, SP limit 10 °C, soaked in 3% H<sub>2</sub>O<sub>2</sub> methanol solution for 5 min, then 15 min in 1  $\times$  Universal blocking solution (Bio-Genex, CA, United States) and

**Table 1** Primers for real-time polymerase chain reaction

Gene	Genbank no	Forward primer (5' to 3')	Reverse primer (5' to 3')
TGF $\beta$ 1	NM_011577	TGCTAATGGTGGACCGCAA	CACTGCTTCCCAGAAATGTCTGA
T $\beta$ R I	NM_009370	ATGGTTCCGAGAGGCAGAGAT	CCATGTCCCATTGTCTTTGTTG
T $\beta$ R II	NM_009371	CCAGAAGTCCTGCAITGAGCAA	TGGCAAACCGTCTCCAGAGTA
Smad2	NM_010754	TCTCCGGCTGAACTGTCTCCTA	GCGATTGAACACCAGAATGCA
Smad3	NM_016769	ATGGAGCTCTGTGAGTTGCCT	TGGAGGTAGAACTGGCGTCTCT
$\alpha$ -SMA	NM_007392	CTATTCAGGCTGTGCTGTCCCT	GCCCTCATAGATAGGCACGTTG
$\alpha$ 1(I) collagen	NM_007742	CCCAAGGAAAAGAAGCACGTC	AGGTCAGCTGGATAGCGACATC
GAPDH	NM_008084	AGCCTCGTCCCGTAGACAAAA	TGGCAACAATCTCCACTTTGC

TGF: Transforming growth factor; T $\beta$ R: TGF- $\beta$  receptor; SMA: Smooth muscle actin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

20 min in 10% goat serum to prevent nonspecific staining. After that, sections were incubated with primary antibodies for 1 h at room temperature in a moist chamber. After three washes with 0.1% Tween 20 in PBS (PBST) for 5 min, Envision<sup>TM</sup> (DakoCytomation, Glostrup, Denmark) was applied according to the procedure and counterstained with Mayer's hematoxylin (DakoCytomation) or DAPI (4',6-diamidino-2-phenylindole 2HCl, D9542, Sigma).

### Western blotting

Liver tissue was homogenized in an Ultra-Turrax homogenizer in RIPA buffer containing 1 mmol/L PMSF and protease inhibitors. After high-speed (12700 g) centrifugation at 4 °C, the protein in the supernatant was separated by 10% SDS-PAGE (20  $\mu$ g per lane), and transferred onto a PVDF membrane. After blocking with 1.5% bovine serum albumin (BSA) in Tris-buffered saline, TGF- $\beta$ 1, T $\beta$ R I, T $\beta$ R II, p-Smad2/3,  $\alpha$ -SMA and  $\alpha$ 1 (I) collagen were detected using rabbit polyclonal antibodies against TGF- $\beta$ 1 (1:1000), T $\beta$ R I (1:1000), T $\beta$ R II (1:1000), p-Smad2/3 (1:2000),  $\alpha$ 1 (I) collagen (1:2000), and  $\alpha$ -SMA (1:400), respectively, and then incubated with anti-rabbit and mouse IgG HRP conjugated (Promega, Madison, WI, United States). Specific binding was detected using the Super Signal West Dura Extended Duration Substrate (PIERCE, Rockford, IL, United States) with a FluorTech 8800 gel doc system (Alpha Innotech, CA, United States) equipped with a chemiluminescent filter.

### Real-time PCR

Total RNA was isolated using TRI-Reagent (Sigma). Real-time PCR was carried out as described<sup>[14]</sup>. DNase I-treated total RNA (1  $\mu$ g) was used for synthesis of the first strand of cDNA. Reverse transcription conditions were as follows: 42 °C for 15 min, 95 °C for 5 min and 5 °C for 5 min (one cycle). Real-time PCR was carried out in 25  $\mu$ L of reaction solution (2.5  $\mu$ L of 10  $\times$  buffer, 5 mmol/L of each dNTP, 10 mmol/L MgCl<sub>2</sub>, 200 nmol/L primers and 0.75 unit of platinum<sup>®</sup> Taq polymerase; all from Invitrogen) plus 1  $\mu$ L of SYBR Green (1:2000; BioWhittaker, Richland, ME, United States). No genomic DNA contamination or pseudogenes were detected by PCR in the absence of the reverse transcription step in

the total RNA used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an invariant control. The reactions started at 95 °C for 7 min, followed by 40 cycles of 95 °C for 20 s, 54 °C for 30 s and 72 °C for 30 s. Melting peaks of PCR products were determined by heat denaturation from 60 to 95 °C at 0.2 °C/s. Fold changes in the mRNA levels of target genes relative to the endogenous GAPDH control were calculated as suggested by Schmittgen *et al.*<sup>[15]</sup>. Table 1 lists the primers used in real-time PCR.

Real-time PCR was performed for quantitative analyses according to standard protocol using the SYBR Green PCR Master Mix and ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Tokyo, Japan).

### Flow cytometry

Livers were first perfused with PBS containing 0.2% BSA, passed through a nylon mesh, and resuspended in PBS/0.2% BSA (EMD chemicals, Gibbstown, NJ, United States). Hepatocytes were removed as pellets after centrifugation at 700 r/min for 60 s periods<sup>[16]</sup>. Lymphocytes from suspended liver cells were then isolated using Histopaque-1077 (Sigma Chemical Co. St. Louis, MO, United States). After centrifugation, cells were washed with PBS/0.2% BSA, and the viability of cells confirmed by trypan blue dye (Sigma Chemical Co. St. Louis, MO, United States) exclusion. Cell preparations were then pre-incubated with anti-mouse FcR blocking reagent and then incubated at 4 °C with a combination of fluorochrome-conjugated antibodies, including anti-CD4 FITC, anti-CD25 APC, anti-CD8 PECy5, anti-Foxp3 PE (all from eBioscience CA, United States). Multiple-color flow analyses were performed using a FACScan flow cytometer upregulated by Cytec Development (Fremont, CA, United States) to allow for 4-color analysis. Acquired data were analyzed with CELLQUEST Software (BD Biosciences CA, United States) and FlowJo Software (Tree star, Inc., Ashland, OR, United States).

### Statistical analysis

Results are expressed as mean  $\pm$  SD and were evaluated using Mann-Whitney *U* tests for comparison between samples from mouse model and littermates, with *P* < 0.05 considered significant.

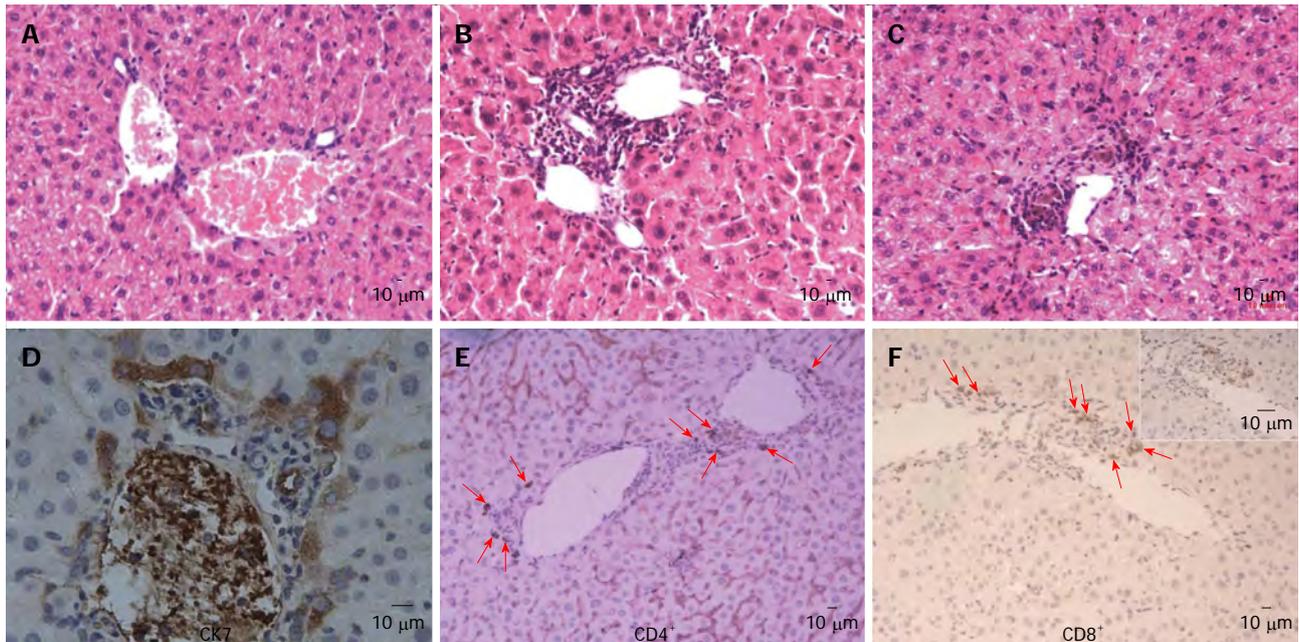


Figure 1 Histological features of the liver. A: Control mice; B-F: Mice model; B: Lymphocytic infiltration (red arrows) around the small bile ducts within the portal tracts at week 8; C: Bile plugs were seen in canaliculi at week 24; D: CK-7 expression in periportal proliferated bile ductile and intralobular hepatocytes; E: CD4<sup>+</sup> lymphocytes infiltration; F: CD8<sup>+</sup> lymphocytes infiltration (bar 10  $\mu$ m).

## RESULTS

### Histological features in Poly I : C induced animal model

The serum levels of ALT, ALP and total bilirubin in the mouse model were higher than in the control mice ( $105.5 \pm 36.9$  IU/L *vs*  $28.2 \pm 2.9$  IU/L,  $P = 0.006$ ;  $138.2 \pm 15.3$  IU/L *vs*  $74.8 \pm 18.5$  IU/L,  $P = 0.025$ ; and  $2.8 \pm 0.4$  mg/dL *vs*  $0.95 \pm 0.12$  mg/dL,  $P = 0.043$ ). Mouse model displayed an increase AMA titer over time. By week 24, serum samples of the six mouse models were all positive for AMA/M<sub>2</sub>. In the mouse model, the mean titer of anti-M<sub>2</sub> was significantly higher at week 24 than at week 8 ( $P < 0.0005$ ), while in the control mice AMA/M<sub>2</sub> was not detected. The time table of AMA in the mouse model resembled that in human PBC, of which the disease is not observed in childhood and typically develops in the fourth or fifth decade of life.

In the liver of the mouse model, moderate to severe infiltration of lymphoid cells was detected within the portal tracts in association with bile duct damage and a mild interface hepatitis (piecemeal necrosis) at week 8 (Figure 1B) and bile plugs were seen in canaliculi around portal tracts at week 24 (Figure 1C), which was absent in control mice (Figure 1A). Direct bile duct destruction was determined by the detection of scattered portal infiltration of CK-7 positive cells. Moreover, in liver tissues from some mice models, biliary cell destruction was so severe that identification of an intact bile duct structure was impossible and all biliary-type and hepatocytes were CK-7 positive, particularly in samples with cholestasis (Figure 1D). Immunohistochemical analysis demonstrated infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes around small bile ducts that were absent in control mice (Figure 1E and F).

### In situ detection of TGF $\beta$ 1, T $\beta$ R I, T $\beta$ R II, p-Smad2/3, $\alpha$ -SMA and $\alpha$ 1 (I) collagen in liver

In mouse model, expression of TGF  $\beta$ 1 in periportal and intralobular regions became more prominent over time (Figure 2A-D). At week 8, there were positive expressions of T $\beta$ R I and T $\beta$ R II in some periportal hepatocytes and biliary ductile endothelial cells (Figure 2E-H). At week 24, distribution of T $\beta$ R I and T $\beta$ R II became more extensive and prominent (Figure 2F and I).

In mouse model, intranuclear staining of p-Smad2/3 was observed in some periportal and intralobular hepatocytes at week 8, and became more prominent at week 24 (Figure 3A-C),  $\alpha$ -SMA positive staining and collagen deposition around portal areas were observed at week 8 (Figure 3E and H), and extension into surrounding parenchyma at week 24 (Figure 3F and I), which was absent in the liver of control mice (Figure 3D and G).

### Immunoblot of TGF $\beta$ 1, T $\beta$ R I, T $\beta$ R II, p-Smad2/3, $\alpha$ -SMA and $\alpha$ 1 (I) collagen

Immunoblot analysis of TGF- $\beta$ 1, T $\beta$ R I, T $\beta$ R II, p-Smad2/3,  $\alpha$ -SMA and  $\alpha$ 1 (I) collagen of the liver homogenates from mouse model and control mice at week 8 and 24 is shown in Figure 4. Compared with that from control mice, there were increasing expressions of TGF- $\beta$ 1, T $\beta$ R I, T $\beta$ R II, p-Smad2/3,  $\alpha$ -SMA and  $\alpha$ 1 (I) collagen of the liver homogenates from mouse model as time increased.

### Real-time PCR of TGF $\beta$ 1, T $\beta$ R I, T $\beta$ R II, Smad2/3, $\alpha$ -SMA and $\alpha$ 1 (I) collagen

As shown in Table 2, the mRNA levels of TGF- $\beta$ 1, T $\beta$ R I, T $\beta$ R II, Smad2, Smad3,  $\alpha$ -SMA and  $\alpha$ 1 (I) collagen

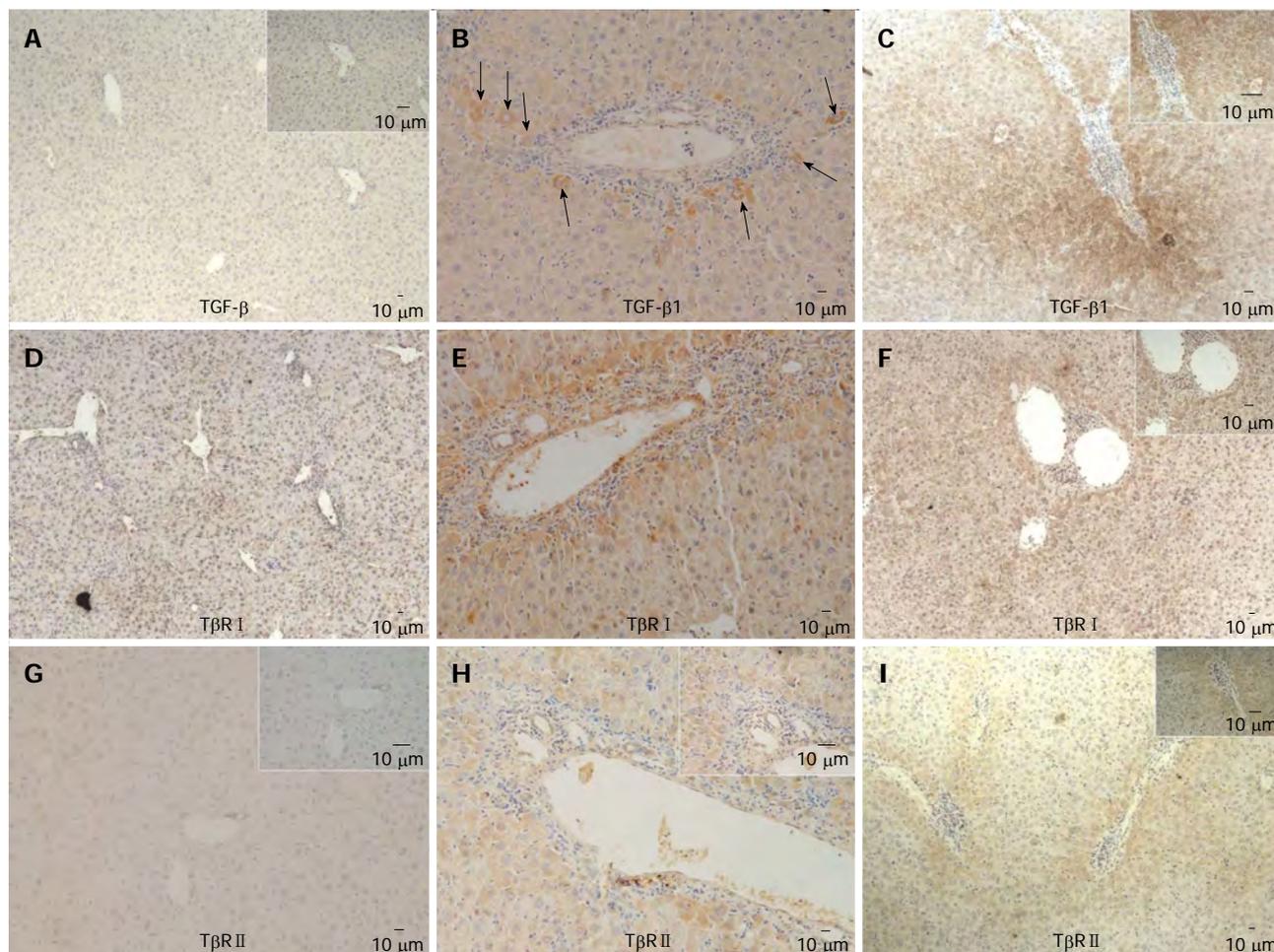


Figure 2 Expressions of transforming growth factor- $\beta$ 1, transforming growth factor- $\beta$  receptor I, transforming growth factor- $\beta$  receptor II in liver. A, D and G: Control mice; B, C, E, F, H and I: Mouse model; A-C: Transforming growth factor- $\beta$ 1 (TGF- $\beta$ ) expression; D-F: TGF- $\beta$  receptor I (T $\beta$ R I) expression; G-I: Transforming growth factor- $\beta$  receptor II expression (bar 10  $\mu$ m).

**Table 2 mRNA levels of transforming growth factor- $\beta$ 1, transforming growth factor- $\beta$  receptor I, transforming growth factor- $\beta$  receptor II, Smad2, Smad3,  $\alpha$ -smooth muscle actin and  $\alpha$ 1 (I) collagen in mouse model and control mice**

	Control mice	Mouse model	
		week 8	week 24
TGF- $\beta$ 1	1.7 $\pm$ 0.4	7.0 $\pm$ 1.8 <sup>b</sup>	8.9 $\pm$ 1.8 <sup>b</sup>
T $\beta$ R I	0.8 $\pm$ 0.2	2.8 $\pm$ 0.7 <sup>b</sup>	5.1 $\pm$ 1.5 <sup>b</sup>
T $\beta$ R II	0.6 $\pm$ 0.01	1.9 $\pm$ 0.9 <sup>b</sup>	5.1 $\pm$ 0.1 <sup>b</sup>
Smad2	0.6 $\pm$ 0.3	3.8 $\pm$ 1.1 <sup>b</sup>	2.0 $\pm$ 0.3 <sup>b</sup>
Smad3	0.9 $\pm$ 0.4	1.7 $\pm$ 0.8 <sup>a</sup>	3.4 $\pm$ 0.6 <sup>b</sup>
$\alpha$ -SMA	0.8 $\pm$ 0.4	1.8 $\pm$ 0.1 <sup>a</sup>	1.7 $\pm$ 0.3 <sup>b</sup>
$\alpha$ 1 (I) collagen	1.1 $\pm$ 1.2	11.0 $\pm$ 1.5 <sup>b</sup>	11.8 $\pm$ 0.6 <sup>b</sup>

The mRNA fold changes were calculated using glyceraldehyde-3-phosphate dehydrogenase as a control. Values were expressed as mean  $\pm$  SD from 3 independent experiments. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 *vs* control mice. TGF: Transforming growth factor; T $\beta$ R: TGF- $\beta$  receptor; SMA: Smooth muscle actin.

of liver homogenates from mouse model at weeks 8 and 24 were higher than that from control mice.

**Flow cytometric analysis of lymphocyte subsets in liver**  
After poly I : C injection, the total numbers of lympho-

cytes significantly increased in the liver of mouse model (Table 3). Although the total number of intrahepatic CD4<sup>+</sup> lymphocytes increased, the percentage of CD4<sup>+</sup> cells in the lymphocytes did not (Figure 5). In contrast, the CD8<sup>+</sup> population in mouse model significantly increased in both total number and percentage compared with that in controls (Figure 5). In addition, the mouse model had a marked increase in the number as well as percentage of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> lymphocytes compared with control mice (Table 3 and Figure 5). This finding is particularly interesting, as previous studies reported a decrease in precursors of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (Treg) in the peripheral blood of PBC patients<sup>[7,17,18]</sup>, and several recent reports demonstrated increased infiltration of FOXP3<sup>+</sup> Treg in damaged organ or target tissues in autoimmune diseases<sup>[19-21]</sup>.

## DISCUSSION

Our study demonstrated that this mouse model mimic several key phenotypic features of human PBC. It had elevated levels of ALP, AMA, portal bile ducts inflammation, and progressive collagen deposition. In human PBC, there is a ten-fold increase in frequency of CD8<sup>+</sup>

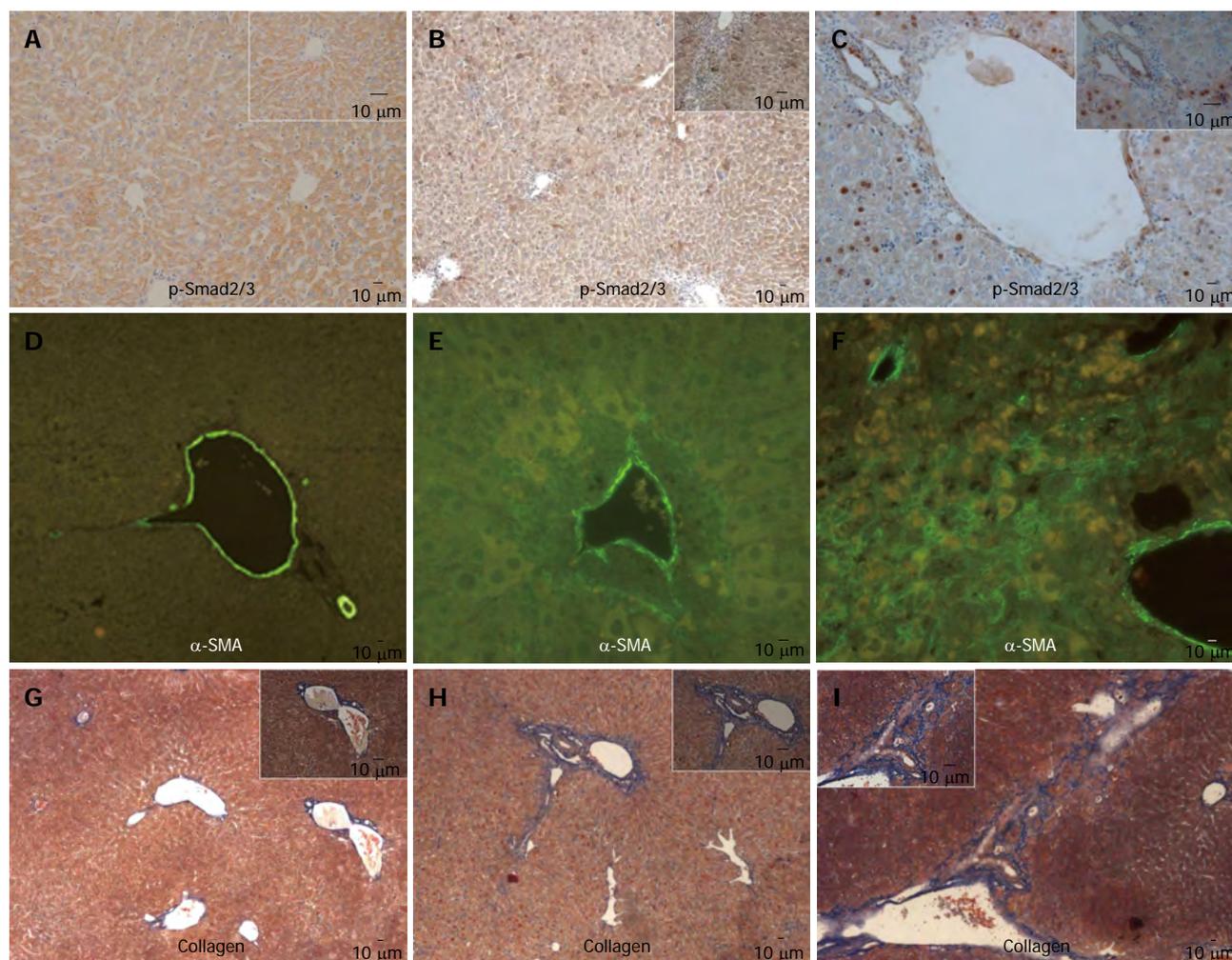


Figure 3 Expression of p-Smad2/3,  $\alpha$ -smooth muscle actin antibody and collagen in liver. A, D and G: Control mice; B, C, E, F, H and I: Mouse model. A-C: p-Smad2/3 expression; D-F:  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibody expression; G-I: Collagen expression (bar 10  $\mu$ m).

**Table 3** Phenotype of mononuclear cells in the liver

	Mouse model	Control mice
Total cell number ( $\times 10^6$ )	$1.6 \pm 0.47^a$	$0.48 \pm 0.32$
CD4 <sup>+</sup> ( $\times 10^6$ )	$0.06 \pm 0.01$	$0.04 \pm 0.01$
CD8 <sup>+</sup> ( $\times 10^6$ )	$0.58 \pm 0.11^a$	$0.08 \pm 0.03$
CD4 <sup>+</sup> /CD8 <sup>+</sup>	$0.12 \pm 0.04^a$	$0.52 \pm 0.23$
CD4 <sup>+</sup> CD25 <sup>+</sup> FOXP3 <sup>+</sup> ( $\times 10^6$ )	$0.01 \pm 0.001^b$	$0.004 \pm 0.001$

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control mice.

T cells specific for PDC-E2 in liver compared with that in peripheral blood, and it correlates with biliary ductular damage<sup>[18,22,23]</sup>. Interestingly, our mouse model also had increased CD8<sup>+</sup> lymphocyte infiltration in liver, which is consistent with the chronic autoimmune nature of the disease. CK-7 is regarded as a histological marker for progression in PBC and indicates poor prognosis<sup>[24]</sup>. Hepatocytes do not normally express CK-7 except in the advanced stage of PBC, which was also observed in our study. Taken together, this animal model had several key phenotypic features and would allow us to analyze the early cellular events of PBC.

TGF- $\beta$ 1 is the key regulator in the pathogenesis of hepatic fibrosis, and appears to aggregate in the liver of PBC patients<sup>[25,26]</sup>. The selective abnormality of the TGF- $\beta$ 1 signaling pathway in T lymphocytes leads to impairment to peripheral tolerance and spontaneously development of features characteristic of PBC<sup>[7]</sup>. TGF- $\beta$ 1 is an essential modulator of Foxp3 expression in Tregs cells<sup>[20]</sup>, conditioning their suppressive function. Recent studies have demonstrated reduction in the number of circulating Tregs in patients with PBC<sup>[21]</sup>. In addition, it is reported that the population of Tregs coexpressing Foxp3 and TGF- $\beta$ 1 decreases with age in female NOD mice<sup>[27]</sup>. Tregs produce elevated levels of TGF- $\beta$ 1, and the fact that TGF- $\beta$ 1 signaling receptors are up-regulated on the membrane of Tregs, underscores the potential for autocrine and/or paracrine receptor-ligand interaction in these cells. TGF- $\beta$ 1 is a positive regulator of Tregs expansion and inhibits autoimmune diseases *via* regulation of the size of Tregs pool *in vivo*<sup>[28]</sup>. Our study found elevated levels of TGF- $\beta$ 1 as well as the total number of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg in the liver of mouse model, which seems different from some studies<sup>[29-31]</sup>. However, there were also several reports demonstrating increased

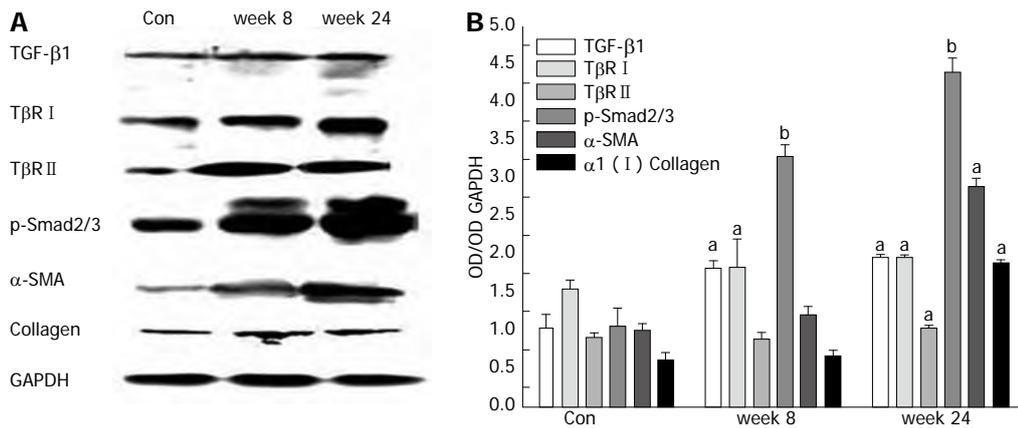


Figure 4 Immunoblot of transforming growth factor- $\beta$ 1, transforming growth factor- $\beta$  receptor I, transforming growth factor- $\beta$  receptor II, p-Smad2/3,  $\alpha$ -smooth muscle actin antibody and  $\alpha$ 1 (I) collagen. A: Western blotting analyses of transforming growth factor (TGF)- $\beta$ 1, TGF- $\beta$  receptor I (T $\beta$ R I), T $\beta$ R II, pSmad2/3,  $\alpha$ -smooth muscle actin (SMA) antibody and  $\alpha$ 1 (I) collagen expression of the liver homogenates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was an internal control for equal loading ( $n = 6$ ); B: <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control mice.

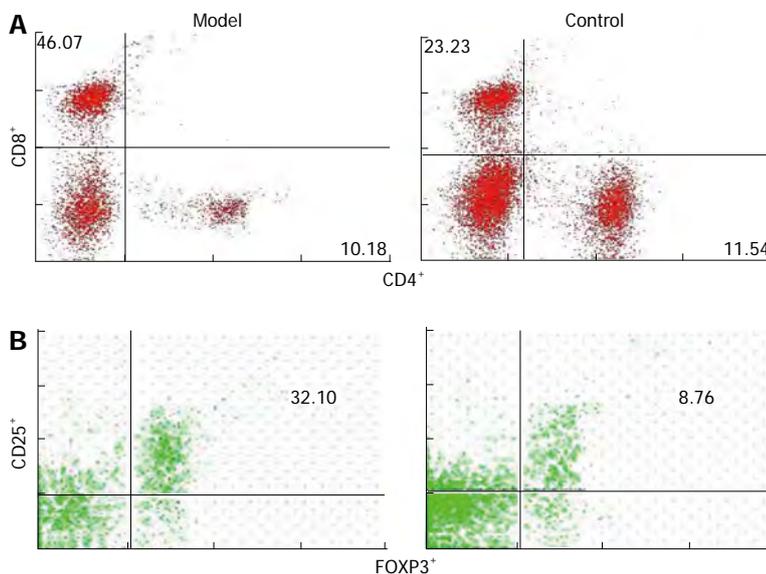


Figure 5 Lymphocytic subsets of the liver. A: The percentage of CD4<sup>+</sup> and CD8<sup>+</sup> cells in total lymphocytes population in liver; B: The percentage of CD25<sup>+</sup> FOXP3<sup>+</sup> in CD4<sup>+</sup> cells population in liver.

infiltration of FOXP3<sup>+</sup> Tregs in damaged organ or target tissues in autoimmune diseases, suggesting that suppressor cells migrate to and/or multiply at the sites of inflammation as part of immune response to combat injurious inflammation<sup>[19-21]</sup>, and in liver suppress hepatic immunity to autoantigens<sup>[32]</sup>. Taken together, our study illustrates that TGF- $\beta$ 1 regulation of FOXP3<sup>+</sup> Tregs may be involved in the maintenance of chronic inflammation in PBC.

TGF- $\beta$ 1 down-regulates potentially harmful inflammatory responses in the liver, albeit at the expense of scar formation<sup>[33]</sup>. TGF- $\beta$ 1 signaling could induce phosphorylation of Smad2 and Smad3, which translocate into the nucleus to regulate expressions of specific target genes such as  $\alpha$ 1 (I) collagen and  $\alpha$ -SMA<sup>[34]</sup>. Our study demonstrated that in the liver of mouse model, the levels of TGF $\beta$ 1 as well as T $\beta$ R I, T $\beta$ R II, p-Smad2/3,  $\alpha$ -SMA

and  $\alpha$ 1 (I) collagen increased with age. These findings revealed that TGF $\beta$ 1 may be involved in the fibrogenesis of the mice PBC model. Liver fibrosis occurs as a consequence of the differentiation of hepatic stellate cells (HSCs) into myofibroblasts, which is regulated by TGF $\beta$ 1<sup>[35]</sup>. Our study showed that the number of cells positive for  $\alpha$ -SMA, which is a marker for myofibroblast-like cells<sup>[36]</sup>, increased in aged mice in the animal model, which was coincident with increased expression of TGF $\beta$ 1 and its signal molecules, supporting the finding that TGF $\beta$ 1 signal pathway was involved in myofibroblast differentiation and subsequent liver fibrosis in the mouse PBC model.

In conclusion, although our data are derived from a murine model of PBC whose immunoregulation in PBC is likely to be far less complex than in human, the findings emphasize the role of TGF $\beta$ 1 in development of

PBC. TGF $\beta$ 1 plays a dual role in development of PBC: it suppresses inflammatory response but operates to enhance fibrogenesis. The aberrant activity of TGF- $\beta$ 1 signaling contributes to the development of PBC.

## ACKNOWLEDGMENTS

We thank Dr. Wei-Xun Zhou for the histological studies.

## COMMENTS

### Background

Primary biliary cirrhosis (PBC) is an autoimmune liver disease. Recent studies suggest that transforming growth factor (TGF)- $\beta$ 1 signaling pathway might play an important role in the pathogenesis of PBC. However, whether TGF- $\beta$ 1 signaling pathway is involved in the development of PBC is still unknown.

### Research frontiers

TGF- $\beta$ 1 plays an important role in autoimmunity and liver fibrosis, and a TGF- $\beta$ 1 receptor knockout mouse has been recently proposed as a model for PBC. There is strong experimental evidence that TGF- $\beta$ 1 is implicated in the pathogenesis of PBC, probably through deregulation of T-reg.

### Innovations and breakthroughs

An animal model of PBC was developed by polyinosinic polycytidylic acids (poly I :C) injection in genetically susceptible C57BL/6 female mice in this study. And the liver expressions of TGF- $\beta$ 1, TGF- $\beta$  receptor I (T $\beta$ R I), T $\beta$ R II, p-Smad2/3, monoclonal  $\alpha$ -smooth muscle actin antibody ( $\alpha$ -SMA) and  $\alpha$ 1(I) collagen in mouse model and control mice were evaluated. The relationship between TGF- $\beta$  and Treg was also analyzed. The study found that TGF $\beta$ 1 played a dual role in the development of PBC. The aberrant TGF- $\beta$ 1 signaling contributed to the development of PBC.

### Applications

This study has provided new data of TGF- $\beta$ 1 signaling pathway involving the pathogenesis of PBC, which will pose significant impact on the understanding of the pathogenesis of PBC. Moreover, the data is the novel result of the role of TGF- $\beta$ 1 in the development of PBC. TGF- $\beta$ 1 signaling pathway is a potential target for PBC treatment.

### Peer review

This paper finds that aberrant TGF- $\beta$ 1 signaling contributes to the development in PBC. Until now we do not have a good answer for the role of TGF- $\beta$ 1 signaling in PBC. These findings may be related to the immunological abnormalities of PBC while the role of TGF- $\beta$ 1 signaling needs further investigation.

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## Consumption of gluten with gluten-degrading enzyme by celiac patients: A pilot-study

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### Abstract

**AIM:** To assess the safety and efficacy of *Aspergillus niger* prolyl endoprotease (AN-PEP) to mitigate the im-

munogenic effects of gluten in celiac patients.

**METHODS:** Patients with initial diagnosis of celiac disease as confirmed by positive serology with subtotal or total villous atrophy on duodenal biopsies who adhere to a strict gluten-free diet (GFD) resulting in normalised antibodies and mucosal healing classified as Marsh 0 or I were included. In a randomised double-blind placebo-controlled pilot study, patients consumed toast (approximately 7 g/d gluten) with AN-PEP for 2 wk (safety phase). After a 2-wk washout period with adherence of the usual GFD, 14 patients were randomised to gluten intake with either AN-PEP or placebo for 2 wk (efficacy phase). Measurements at baseline included complaints, quality-of-life, serum antibodies, immunophenotyping of T-cells and duodenal mucosa immunohistology. Furthermore, serum and quality of life questionnaires were collected during and after the safety, washout and efficacy phase. Duodenal biopsies were collected after the safety phase and after the efficacy phase. A change in histological evaluation according to the modified Marsh classification was the primary endpoint.

**RESULTS:** In total, 16 adults were enrolled in the study. No serious adverse events occurred during the trial and no patients withdrew during the trial. The mean score for the gastrointestinal subcategory of the celiac disease quality (CDQ) was relatively high throughout the study, indicating that AN-PEP was well tolerated. In the efficacy phase, the CDQ scores of patients consuming gluten with placebo or gluten with AN-PEP did not significantly deteriorate and moreover no differences between the groups were observed. During the efficacy phase, neither the placebo nor the AN-PEP group developed significant antibody titers. The IgA-EM concentrations remained negative in both groups. Two patients were excluded from entering the efficacy phase as their mucosa showed an increase of

two Marsh steps after the safety phase, yet with undetectable serum antibodies, while 14 patients were considered histologically stable on gluten with AN-PEP. Also after the efficacy phase, no significant deterioration was observed regarding immunohistological and flow cytometric evaluation in the group consuming placebo compared to the group receiving AN-PEP. Furthermore, IgA-tTG deposit staining increased after 2 wk of gluten compared to baseline in four out of seven patients on placebo. In the seven patients receiving AN-PEP, one patient showed increased and one showed decreased IgA-tTG deposits.

**CONCLUSION:** AN-PEP appears to be well tolerated. However, the primary endpoint was not met due to lack of clinical deterioration upon placebo, impeding an effect of AN-PEP.

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**Key words:** Celiac disease; Gluten; Enzyme; Prolyl endoprotease; *Aspergillus niger* prolyl endoprotease; Treatment; Adverse events; efficacy; IgA-tTG intestinal deposits

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## INTRODUCTION

Celiac disease (CD) is a major health care issue affecting people of all ages, with a worldwide prevalence of approximately 1%<sup>[1]</sup>. This immune-mediated small intestinal enteropathy is triggered by gluten proteins derived from wheat, barley and rye. Celiac disease is characterised by an inflammatory immune response, resulting in small-intestinal mucosal injury and malabsorption in genetically susceptible individuals<sup>[2]</sup>. Currently, the only safe and effective treatment is a strict gluten-free diet (GFD) combined with nutritional support, which improves the health and quality of life in the vast majority of patients<sup>[3]</sup>. However, a GFD is perceived as a substantial burden, particularly due to high costs, dietary restriction, reduced social activity, and increased health worries<sup>[4]</sup>.

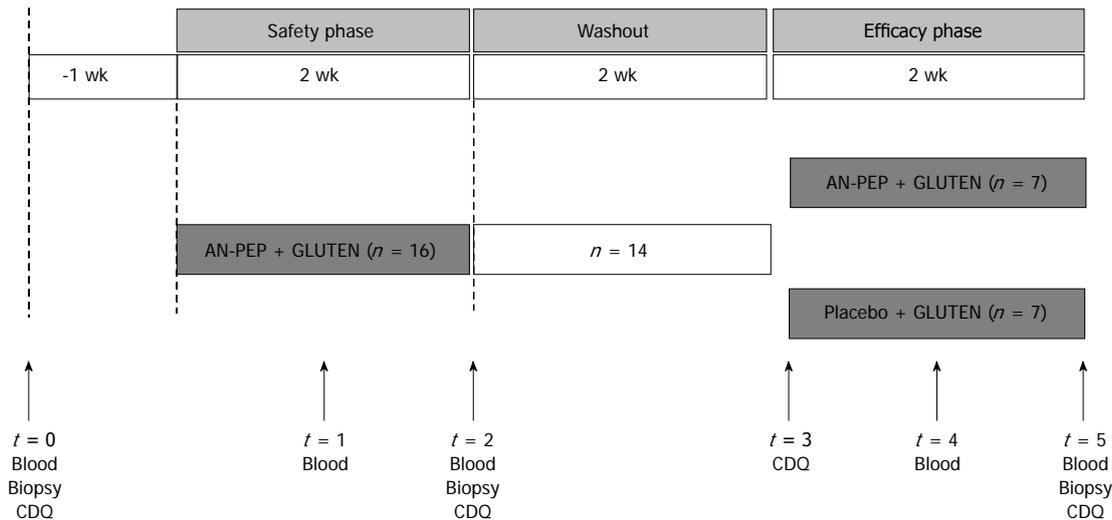
Gluten proteins are highly abundant in proline (15%) and glutamine (35%) residues, particularly in those regions identified as immunogenic in CD<sup>[5]</sup>. The proline- and glutamine-rich peptides in gluten are relatively resistant to proteolysis by gastric, pancreatic and intestinal enzymes<sup>[6,7]</sup>. Consequently, digestion-resistant proline- and glutamine-rich peptides can reach the intestinal epi-

thelium intact and can trigger an immune response that eventually results in mucosal damage. To eliminate such proline-rich gluten peptides, prolyl oligopeptidases, enzymes that can cleave after a proline residue in peptides, have been investigated by Shan and colleagues<sup>[6]</sup>. Such enzymes, derived from bacteria like *Flavobacterium meningoseptum*, *Sphingomonas capsulate* and *Myxococcus xanthus*, were capable of breaking down toxic gluten *in vitro*<sup>[6,8,9]</sup>. These prolyl oligopeptidases are however not stable and functional under acidic conditions of the stomach<sup>[9,10]</sup> and are unlikely to degrade gluten epitopes before they reach the small intestine. Alternative enzymes that can break down gluten are derived from germinating barley and the fungus *Aspergillus niger*. From the latter a prolyl endoprotease termed *Aspergillus niger*-derived prolyl endoprotease (AN-PEP) is derived which has distinct advantages over the bacterial prolyl oligopeptidase as it degrades both whole gluten and gluten peptides into non-immunogenic residues within minutes<sup>[11,12]</sup>. Moreover, the enzyme is active between pH 2 and pH 8, with an optimum activity at pH 4-5, and is therefore effective at the pH levels present in the stomach and beyond<sup>[11,13]</sup>. Importantly, the enzyme is not degraded by pepsin in the stomach and thus remains fully functional. Mitea *et al*<sup>[12]</sup> extended these findings by showing that AN-PEP degraded toxic gluten proteins in a food matrix into non-immunogenic gluten fragments in an *in vitro* digestion model that simulates the human gastrointestinal tract. After these promising *in vitro* results, it remains to be established in CD patients whether AN-PEP can reduce the clinical response to gluten. The aim of this two-phase proof of concept study was to demonstrate the safety of AN-PEP in the first phase and the ability of ANPEP to reduce antibody and histological response to gluten consumption by CD patients in the second phase of the study. This information will be important to further develop AN-PEP as a future digestive aid for unintentional ingestion of gluten by CD patients.

## MATERIALS AND METHODS

### Patients

Sixteen adults with CD were recruited at the outpatient clinic of the department of Gastroenterology and Hepatology of the VU Medical Centre Amsterdam, The Netherlands. Inclusion criteria were an initial diagnosis of CD as confirmed by histological abnormalities on duodenal biopsies classified as a Marsh III B or III C lesion and supported by positive serology; endomysium IgA antibodies (IgA-EM) and/or tissue transglutaminase IgA antibodies (IgA-tTG). Patients were required to have well-controlled CD as evidenced by Marsh 0 or I, and normalised IgA-EM and IgA-tTG on a strict GFD for at least one year. Women at fertile age were required to take adequate contraception measures. Reasons for exclusion were: use of any anticoagulant or immunoregulatory drug within the last 6 mo; clinically suspected bleeding tendency; pregnancy or breast feeding; presence of any concurrent active infection; and IgA deficiency.



**Figure 1 Study design and flowchart.** In the safety phase, 16 patients daily consumed 5 pieces of toast with *Aspergillus niger* prolyl endoprotease (AN-PEP) for 2 wk while continuing their gluten-free diet (GFD). Two patients deteriorated on Marsh scores and were excluded. After a 2-wk wash-out period during which the patients continued their usual GFD, the remainder of 14 patients were randomized to the efficacy phase to receive 2 wk of toast with either AN-PEP or placebo while continuing their GFD. CDQ: Celiac disease quality.

### Design and intervention

The intervention was performed between May 2008 and April 2009. The intervention consisted of two periods, each lasting 2 wk (Figure 1). The first study phase was an open-label period designed to assess the safety of high gluten intake with AN-PEP (safety phase). The second phase was a randomised, double-blind, placebo-controlled parallel-group study to assess the effect of AN-PEP on gluten-induced clinical response (efficacy phase). Sixteen patients with diagnosed CD were enrolled in the safety phase. Patients were asked to consume five pieces of toast (in total approximately 7 g gluten, Bolletje<sup>®</sup>, The Netherlands) with AN-PEP-containing topping daily in the morning for 2 wk. Patients were allowed to consume a glass of water (250 mL) with their toast. They were asked to continue their usual GFD. For ethical reasons, patients deteriorating  $\geq 2$  scales on the histological Marsh classification during this safety phase were not included in the efficacy phase. Between the study phases, a 2-wk washout period was introduced in which patients continued their usual GFD. Subsequently, fourteen patients were randomised in a 1:1 ratio in blocks of four in a double-blind fashion to the same amount of toast with AN-PEP-containing topping ( $n = 7$ ) or placebo topping ( $n = 7$ ) for 2 wk while remaining on their usual GFD. Patients' compliance with the product intake was checked by regular telephone contact.

Before and during the study phases, the patients visited the outpatient clinic five times (Figure 1). During the safety phase, blood was collected one week before (baseline), and one and two wk after start of gluten with AN-PEP consumption. During the efficacy phase, blood was collected at one and two wk after start of gluten with AN-PEP or placebo consumption. Duodenal biopsies were taken at baseline and at the end of the safety phase and the end of the efficacy phase. Both in the safety and

efficacy phase, participants were asked to complete a celiac disease-specific health-related quality of life questionnaire for adults<sup>[14]</sup> at baseline and after two wk of intervention. Biopsies and blood sampled at the end of the safety phase were used as baseline values to limit the burden for the patients.

### AN-PEP enzyme

The AN-PEP and placebo topping were prepared by DSM Food Specialties, Delft, The Netherlands. Both toppings (18.5 g) contained 8.2 wt% sucrose, 8.2 wt% saccharine solution (400 mg/L saccharine plus 4000 mg/L cyclamate), 0.4 wt% citric acid (Jungbunzlauer, Basel, Switzerland), 0.08 wt% potassium sorbate (Interland Chemie, Oosterhout, The Netherlands), 0.31 wt% sodium benzoate (Prolabo, Leuven, Belgium), and 1.23 wt% xanthane gum Keltrol RD (CP Kelcko, Nijmegen, The Netherlands). The AN-PEP topping contained 81.5 wt% AN-PEP enzyme concentrate corresponding with 168 Proline Protease Units of enzyme activity. The placebo topping contained 81.5 wt% distilled water with 0.06 wt% Plantex<sup>®</sup> MDA31 (colouring agent, DSM Food Specialties, Delft, The Netherlands) to match for colour differences. The aroma, flavour and consistency of the topping with AN-PEP were identical to those with placebo and both toppings could not be distinguished. Microbial counts and enzyme activity of the AN-PEP and placebo toppings were analysed monthly. All microbial counts remained below 10 CFU/g and the activity of the enzyme was maintained at  $9.1 \pm 0.3$  PPU/g topping during 12 mo shelf life at 4 °C. The AN-PEP and placebo toppings were identical in taste and appearance. They were pre-packed in containers (14 per box) by DSM and consecutively numbered for each patient according to the randomisation schedule (prepared by the DSM statistician).

**Blinding**

Each patient was assigned a random order number and received from the physician the containers in the corresponding non-transparent pre-packed box. The allocation sequence was concealed from the researcher enrolling and assessing participants in sequentially numbered sealed non-transparent envelopes. Envelopes were opened only after completion of the trial and assessments. All patients, investigators, care providers, and staff assessing outcomes were kept blind to treatment assignment.

**Measurements**

Mucosal biopsy immunohistology and immunophenotyping of lymphocytes, and serum antibodies were measured in the service laboratories of the VU University Medical Centre (Amsterdam, The Netherlands). Mucosal biopsy gluten-specific T-cell lines were measured in the research laboratory of the Leiden University Medical Centre (Leiden, The Netherlands). Mucosal biopsy IgA-tTG deposits were analysed at the University of Debrecen (Hungary).

**Adverse event reporting:** Tolerability of the gluten intake with AN-PEP or placebo was assessed by adverse event reporting to the physician during visits. All complaints were documented throughout the study. The study design did not allow for differentiation between complaints resulting from gluten or treatment. A difference in complaints between the AN-PEP and placebo group during the efficacy phase may give an indication of treatment-related effects.

**Celiac-disease quality of life:** All participants were asked to complete at home the CD quality of life questionnaire, which was translated into Dutch. The CD quality of life questionnaire included four disease-specific and health-related categories (emotional problems, social problems, disease-related worries, and gastrointestinal symptoms) with 7 items each<sup>[14]</sup>. Each question was weighed on a scale of 1-7 points, a high score corresponding to a high level of well-being. In total 196 points could be obtained, with a maximum of 49 points for each separate category. A change of 12 or more points on the total score or of 3 or more on the different categories was considered a clinically relevant change<sup>[14]</sup>.

**Mucosal biopsy immunohistology:** Twelve duodenal mucosal spike biopsies were taken through upper gastrointestinal endoscopy. Four paraffin-embedded biopsies were sectioned and hematoxylin-eosin-stained for histological evaluation according to the modified Marsh classification<sup>[15]</sup>. At least two grades increase in the Marsh scale was considered a clinically significant deterioration. Six fresh biopsies were used for flow cytometric analysis and two were snap frozen in liquid nitrogen and stored.

**Mucosal biopsy immunophenotyping of lymphocytes:** Multiparameter flow cytometric immunophenotyping of mucosal intraepithelial and lamina propria lymphocytes was performed. These lymphocytes were isolated from six duodenal biopsy specimens per time point through chemical and enzymatic dissociation<sup>[16]</sup>. The cells were stained with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein and allophycocyanin-labelled monoclonal antibodies directed against CD3, CD4, CD8, CD16/56, CD19, CD45, CD45RA, HLA-DR, NKG2D, CD25 and TCR gamma-delta (all from BD Biosciences, San Jose, CA, United States), and appropriate isotype controls were included. Stained cells were analysed on a 4-colour flow cytometer (FACSCalibur™, BD Biosciences) and the data were analysed using Cellquest™ software (Becton Dickinson, San Jose, CA, United States). Care was taken to analyse only viable cellular events based on light scatter properties. The mean fluorescence intensity index as compared to isotype controls was calculated for the markers included.

**Mucosal biopsy gluten-specific T-cell lines:** Gut-resident, gluten-reactive T-cells are a hallmark of CD. To demonstrate that all patients possessed such cells, polyclonal T-cell lines were generated from small intestinal biopsies as described<sup>[17]</sup>. The resulting T-cell lines were tested for reactivity against a pepsin/trypsin digest of gluten and a pepsin/trypsin digest of gluten that had been treated with tissue transglutaminase in a T-cell proliferation assay as described<sup>[17]</sup>. In all patients gluten reactivity could be demonstrated (not shown).

**Mucosal biopsy IgA-tTG deposits:** Biopsies at the end of the randomisation study phase were stained for tTG-related extracellular IgA deposits and, in case of positivity, baseline biopsies were stained as well. Twelve unfixed, 5 µm-thick frozen sections were examined per patient by double immunofluorescent labelling of IgA (green) and tTG (red) as previously described<sup>[18]</sup>. IgA is normally detected only inside plasma cells and at the luminal surface, whereas in active CD, subepithelial deposits composed of IgA-tTG are found along the surface and crypt basement membranes and around mucosal vessels, corresponding to the intestinal localisation of tTG. The CD-type IgA-tTG deposits were graded from 0 to 3 according to their intensity along the basement membranes in the villous-crypt area. As this study of the small intestinal IgA-tTG deposits is highly subjective, it was performed by an independent specialist in this field in a blind manner to greatly increase its accuracy.

**Serum antibodies:** Blood samples were collected by venipuncture to analyse CD-associated antibodies. Levels of IgA-tTG, gliadin IgA antibodies (IgA-AG) and gliadin IgG antibodies (IgG-AG) were determined with a standard in house enzyme-linked immunosorbent assay (ELISA), using recombinant human tissue transglutamin-

**Table 1** Demographic and baseline characteristics of the safety and efficacy phase

	Safety phase	Efficacy phase	
	Gluten + AN-PEP ( <i>n</i> = 16)	Gluten + placebo ( <i>n</i> = 7)	Gluten + AN-PEP ( <i>n</i> = 7)
Patients ( <i>n</i> )	16	7	7
Gender (female:male)	12:4	5:2	6:1
Median age at inclusion, yr (range)	55 (20-68)	44 (20-68)	57 (30-64)
Median age at diagnosis, yr (range)	44.5 (0-62)	29 (0-62)	49 (26-53)
Median time on a GFD, yr (range)	7.5 (2-40)	9 (2-40)	8 (4-12)
HLA class ( <i>n</i> )			
DQ2/X	12	5	5
DQ2/DQ2	2	1	1
DQ2/DQ8	1	0	1
Unknown	1	1	0
Marsh at inclusion ( <i>n</i> )			
Marsh 0	10	4	3
Marsh I	6	3	4
Gastrointestinal symptoms			
Abnormal bowel sounds	4	0	2
Abdominal pain	5	3	2
Bowel distension	5	3	1
Change of defecation	4	2	6
Constipation	3	2	0
Diarrhoea	3	1	1
Dysgeusia	1	1	0
Flatulence	6	1	2
Nausea	4	2	0
Reflux	2	0	1
Vomiting	1	1	0
Weight loss	0	1	0
Total number of symptoms	38	17	15

GFD: Gluten-free diet; AN-PEP: *Aspergillus niger* prolyl endoprotease.

ase (Diarect AG, Freiburg, Germany) and gliadin extract (Sigma-Aldrich, Zwijndrecht, the Netherlands) as substrates, respectively. IgA-EM antibodies were determined by an in-house indirect immunofluorescence test according to Lerner using monkey oesophagus as substrate<sup>[19]</sup>. IgA deficiency was excluded to avoid false negative serology. In addition, in retrospect a combined test for IgA and IgG antibodies directed against human tissue transglutaminase and deamidated gliadin-derived peptides (IgA/G-DGP-tTG; tTG/DGP Screen ELISA, INOVA Diagnostics, San Diego, United States) was performed<sup>[20]</sup>. Reference values for antibodies were categorized into negative, dubious, weak positive, positive, and strong positive. Reference ranges for IgA-AG were < 2.4, 2.5-3.9, 4.0-20, 20-80, and > 81 U/mL, for IgG-AG, < 11, 12-20, 21-40, 41-100 U/mL, for IgA-tTG, < 2.9, 3.0-5.9, 6.0-20, 21-50, > 51 U/mL, and for IgA/G-DGP < 6.9, 7.0-10.9, 11-30, 31-100 and > 100 U/mL respectively.

### Ethical approval

The study was approved by the Medical Ethics Committee of the VU Medical Centre and conducted in accordance with the guidelines of the Declaration of Helsinki. The trial has been registered in the Dutch Trial register (NTR1281) and the FDA Clinical Trial register (NCT00810654). A written informed consent was obtained from each subject before enrolment.

### Statistical analysis

Data were analysed by OCS Biometric Support (Leiden, The Netherlands). Difference from baseline in mucosal immunohistology between the two groups after 2 wk as measured by Marsh classification was considered the primary outcome measure. All other parameters were considered secondary endpoints. Power analysis revealed that for the detection of a two-grade difference in the Marsh score with a power of 0.80 and a one-sided *a* level of 0.05, 14 patients were needed to finalise the study. Data were analysed in the SAS version 9.1, using both parametric and non-parametric tests depending on the nature of the data. The quality of life data were analysed with paired *t* tests to test for differences between data before and after the 1<sup>st</sup> (safety) and 3<sup>rd</sup> (efficacy) period of the study. Serological and histopathological outcome parameters were analysed with Wilcoxon signed-rank tests to determine differences between data before and after the 1<sup>st</sup> period and the Wilcoxon rank sum tests to test the treatment differences in change from baseline in the 3<sup>rd</sup> period of the study. In order to explore whether patients' baseline characteristics would predict their response to gluten (and hence to increase the chances of success in a future trial), rank correlations between baseline characteristics and outcome variables were explored in the placebo group using the Spearman Rank Correlation Coefficient (*r*) of the ranked data (analysed by DSM

statistician).

## RESULTS

### *Baseline characteristics*

The demographic and baseline characteristics of the patients are presented in Table 1. In total, 16 adults on a gluten-free diet diagnosed as having CD [median age: 55 (20-68) years] were enrolled in the study. The demographic characteristics of both treatment groups were comparable with exception of the median age at diagnosis of CD, which was 20 years higher in the AN-PEP compared to the placebo group. The median time on GFD treatment was similar in both groups. Two patients were excluded after the safety phase because of a histological deterioration of two and three Marsh grades, respectively, which for one patient returned to normal (Marsh 0) after four weeks of exclusion. The patient that did not return to normal started the study with high IgA/G-DGP-tTG values. However, other CD-related antibodies remained undetectable in these two patients. The remaining 14 patients entered and completed the efficacy phase.

When correlating the patients' baseline characteristics with their response to gluten, highly significant inverse relationships were found between the patients' time since diagnosis or time spent on a GFD and their response to gluten as measured by IgG-AG, IgA-tTG and IgA/G-DGP-tTG, and Marsh scores (data not shown).

### *Adverse events*

No serious adverse events occurred during the trial, patients reported no severe adverse events, and no patients withdrew during the trial. Complaints that were reported during the safety and efficacy phase were of gastrointestinal nature and mostly mild and transient. The number of reported gastrointestinal complaints did not differ between the AN-PEP and placebo group (Table 1).

### *Celiac-disease quality of life*

The mean total scores of the four categories on the CD quality of life were relatively high (145-156 out of a total score of 196) in the total group and throughout both study phases. In the safety phase, the total CD quality of life score significantly ( $P = 0.04$ ) increased by 6 points during gluten with AN-PEP treatment. This increase was however lower than the 12-point increase that is considered a clinically relevant quality of life improvement<sup>[14]</sup>. In the efficacy phase, the individual or total CD quality of life scores of patients consuming gluten with placebo or gluten with AN-PEP did not significantly deteriorate. No differences between the groups were observed. The mean score for the gastrointestinal CD quality of life was relatively high throughout the study, indicating that gluten with AN-PEP was well tolerated.

### *Mucosal biopsy immunohistology*

In the patients receiving gluten plus AN-PEP treatment

in the safety phase, several patients showed variation in Marsh scores but overall no significant change in degree of mucosal damage, as indicated by changes in the Marsh score, was observed (Table 2). Two of 16 patients were excluded from entering the efficacy phase as their mucosa showed an increase of two Marsh steps while 14 patients were considered histologically stable on gluten with AN-PEP. Also after the efficacy phase, no significant deterioration was observed in the group consuming gluten with placebo compared to the group receiving AN-PEP.

### *Mucosal biopsy immunophenotyping of lymphocytes*

Flow cytometric analysis of intestinal lymphocyte subsets showed no significant changes in the expression of the T-cell lineage associated markers CD3, CD4, CD8 and TCR  $\gamma\delta$ , in either the intraepithelial lymphocyte or the lamina propria lymphocyte populations of both treatment groups during the efficacy phase. The mean fluorescence index of the activation markers CD25, HLA-DR, the NK receptor and NKG2D as well as CD45RA, a marker for naïve T-cells, showed no significant change in either group.

### *Mucosal biopsy IgA-tTG antibody deposits*

Mucosal tTG-related extracellular IgA deposits are hypothesised to be an early marker for CD activity<sup>[21]</sup>. Despite a GFD, two of seven patients started with positive staining for IgA-tTG at baseline (Table 2). Compared to baseline, IgA-tTG deposit staining increased after 2 wk of gluten intake in four out of seven patients on placebo. In the seven patients receiving AN-PEP, one patient showed increased and one showed decreased IgA-tTG deposits (Table 2, Figure 2).

### *Serum antibodies*

Serum CD-associated antibodies (IgA-tTG, IgA-EM, IgA-AG, IgG-AG and IgA/G-DGP-tTG) were not detectable in the serum of enrolled patients at baseline (Table 2) except for one patient in which borderline levels of IgA/G-DGP-tTG were detected, which became negative after 2 wk of gluten with AN-PEP consumption. The IgA-tTG, IgG-AG, IgA/G-DGP-tTG, and IgA-EM antibody titers remained negative on gluten with AN-PEP. Three out of sixteen patients developed detectable or borderline IgA-AG levels, while 13 patients remained negative during 2-wk of gluten with AN-PEP (Table 2).

During the efficacy phase, neither the placebo nor the AN-PEP group developed significant antibody titers (Table 2). The median antibody titers after 2 wk gluten intake did not significantly differ between AN-PEP and placebo treatment. The IgA-EM concentrations remained negative in both groups.

## DISCUSSION

The enzyme AN-PEP might possibly assist in digesting

Table 2 Serum antibodies, duodenal immunohistology and tTGA-A antibody deposits in the safety and efficacy phase for all patients

	Baseline						Safety phase 2 wk gluten + AN-PEP						Efficacy phase 2 wk gluten + AN-PEP or placebo											
	Serum			Biopsy			Tx			Serum			Biopsy			Tx			Serum			Biopsy		
	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	IgA-tTG deposits	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	Tx	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	Tx	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	IgA-tTG deposits
1	-	-	-	-	I	ND	A	-	-	0	P	-	-	-	-	I	0	P	-	-	-	-	I	0
2	-	+/-	-	-	0	0	A	-	+	I	P	-	-	++	+	III A	1-2	P	-	-	+	-	III A	1-2
3	-	-	-	-	I	0	A	-	-	I	P	-	-	-	-	III A	1-2	P	-	-	-	-	III A	1-2
4	+/-	-	-	+	I	1	A	-	+	0	P	+	-	-	I	2-3	P	+	-	-	++	-	I	2-3
5	-	-	-	-	0	ND	A	-	-	0	P	-	-	-	0	0	P	-	-	-	-	-	0	0
6	-	-	-	-	0	0	A	-	-	0	P	-	-	-	0	0	P	-	-	-	-	-	0	1
7	-	-	-	-	0	0	A	-	-	0	P	-	-	-	0	0	P	-	-	-	-	-	I	1-2
8	-	-	-	-	I	ND	A	-	-	II	A	-	-	-	-	I	0	A	-	-	-	-	I	0
9	-	-	-	-	0	3	A	-	-	0	P	-	-	-	-	I	1	A	-	-	+	-	I	1
10	-	-	-	-	0	ND	A	-	-	0	P	-	-	-	-	I	1	A	-	-	+	-	I	0
11	-	-	-	-	I	0	A	-	+	II	A	-	-	-	-	III A	1	A	-	-	+	-	III A	1
12	-	-	-	-	I	ND	A	-	-	I	A	-	-	-	-	I	0	A	-	-	-	-	I	0
13	-	-	-	-	0	ND	A	-	-	0	A	-	-	-	-	0	0	A	-	-	-	-	0	0
14	-	-	-	-	0	ND	A	-	-	0	A	-	-	-	-	0	0	A	-	-	-	-	0	0
15	-	-	-	++	0	ND	A	-	-	++	E	-	-	-	-	III A	ND	E	-	-	++	-	III A	ND
16	-	-	-	-	0	ND	A	-	-	-	E	-	-	-	-	0	E	-	-	-	-	-	0	ND

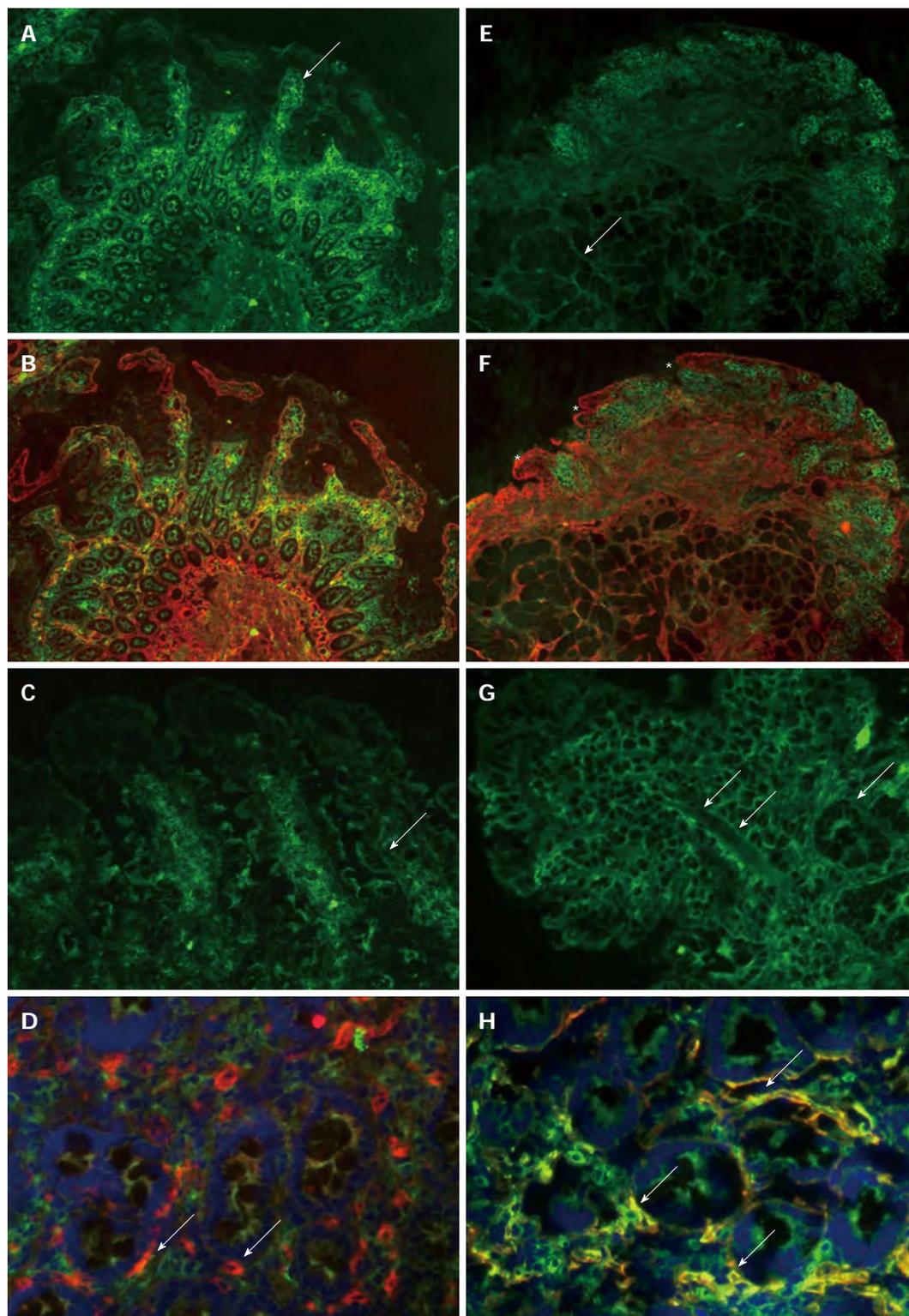
The serum EMA-A antibodies remained negative in all patients during the entire study. Mucosal tTGA-A deposits were graded from 0 to 3. Tx: Treatment; A: AN-PEP; P: Placebo; E: Excluded; IgA-tTG: Anti-tissue transglutaminase IgA antibodies; IgA-GA: Anti-gliadin IgA antibodies; IgG-GA: Anti-gliadin IgG antibodies; IgA/G-DGP-tTG: Anti-tissue transglutaminase and deamidated gliadin-derived peptide IgA and IgG antibodies; I-FABP: Intestinal fatty acid binding protein; ND: Not determined. - : Negative; +/- : Dubious; + : Weak positive; ++ : Positive; +++ : Strong positive.

unintentionally ingested amounts of gluten in those who cannot tolerate gluten. However, demonstrating a treatment effect on (small) clinical deterioration induced by small amounts of gluten in the placebo group may be difficult. Therefore, in this proof of principle study, the enzyme was given to patients consuming large amounts of gluten in a relative small period of time. A two-week safety phase (AN-PEP + gluten) preceded the randomization for AN-PEP or placebo as requested by the medical ethical commission due to concerns about such a high dose of gluten consumption. Unfortunately, the primary aim of the study was not met as the placebo arm did not show any deterioration after 2 wk of gluten consumption. With hindsight, the study should possibly have been designed for a much longer period of time with many more patients.

The baseline characteristics were balanced between groups except for median age at diagnosis, which was 20 years higher in the AN-PEP compared to the placebo group. However, this is unlikely to have influenced the study outcome as no relationship between the age of diagnosis and the response to gluten was observed (data not shown).

The safety phase showed that AN-PEP treatment, when consumed with a high dose of about 7 g of gluten for 2 wk, was safe in patients and no severe adverse events were reported. The CD quality of life scores remained relatively high during 2 wk consumption of gluten and AN-PEP indicating that patients' general well-being remained high. Serum antibodies of the sixteen patients did not increase when consuming AN-PEP with 7 g of gluten for 2 wk. Also, histology of the biopsies of the majority of patients (fourteen) showed no deterioration while two patients developed increased Marsh scores, however not accompanied by increased antibodies. The safety phase was subject to a so-called "ceiling effect" because patients entered the study on a GFD reflecting relatively healthy baseline values, limiting the ability to demonstrate any further improvement by AN-PEP.

Patients in the placebo group did not show significant deterioration on any of the measured clinical variables after a 2-wk gluten challenge, indicating that 2 wk of gluten challenge is insufficient to induce a clear clinical response in this population of celiac patients. Due to lack of response to gluten in the placebo arm, no treatment effect of



**Figure 2 Small intestinal tissue transglutaminase IgA antibody deposits (rated on a scale 0-3) in two patients at baseline and after randomization to *Aspergillus niger* prolyl endoprotease and placebo respectively.** A: Baseline evaluation of patient 1 showed preserved villous architecture (arrow), with intense, grade 3 IgA depositions (green) subepithelially and around crypts; B: This deposition merges to yellow indicating co-localisation with tTG shown in red; C: In this patient, IgA deposits diminished after 2 wk *Aspergillus niger* prolyl endoprotease (AN-PEP) treatment to grade 1, when only faint and patchy antibody deposition was seen (arrow); D: tTG appeared in red in this AN-PEP-treated patient (arrow) in the absence of IgA deposition. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue); E: Baseline evaluation of patient 2 showed preserved villous architecture with faint, grade 1 IgA deposition in the deep mucosal layer around Brunner glands (arrow); F: This deposition were not sufficient to obtain a yellow colour at merging with tTG shown in red (asterisk); G: In this patient, IgA deposition increased to grade 2 subepithelially (arrow) after 2 wk placebo; H: IgA deposition increased to grade 3 in the crypt region after 2-wk placebo (arrow). IgA deposition co-localised with tTG to intense yellow (arrow). The cell nuclei were stained with DAPI (blue).

AN-PEP could be detected. The measured serum levels of IgA-tTG, IgA-EM, and IgA/G-DGP-tTG antibodies are considered sensitive markers of CD and should be able to detect subtle immunogenic effects of gluten. Similarly, the CD-specific quality of life questionnaire is considered a CD-specific measure of quality of life and should also be able to pick up relevant changes in health<sup>[14]</sup>. However, histological examination of small intestinal biopsies may be less reliable than CD-associated antibodies due to heterogeneous distribution of lesions, low grade histopathology, and intra- and interobserver variability<sup>[22]</sup>. Interestingly, measures of clinical response to gluten (Marsh scores, antibody titres, quality of life scores) did not correlate in this study, which may in part be explained by the lack of response to gluten. The IgA antibody reactivity to small intestinal mucosa tTG has been considered to be an early marker for gluten-induced pathology in CD patients<sup>[23]</sup>. It was observed that intestinal IgA-tTG deposits can be detected in latent CD patients in whom the mucosal villous architecture is still intact, and that the intensity of these mucosal deposits decreased after adherence to a GFD and increased after gluten consumption. Although numbers were low, mucosal IgA-tTG deposits increased in four patients on placebo and one on AN-PEP and decreased in one patient on AN-PEP, compared to baseline values, suggesting that AN-PEP may mitigate gluten exposure.

Some gastrointestinal-related symptoms, mostly mild and transient, were reported during gluten challenge and symptoms between the two groups were comparable suggesting no treatment-related effects on gastrointestinal symptoms. Besides the substantial gluten intake, emotional stress as a consequence of having to ingest gluten might have triggered some of the reported gastrointestinal complaints.

The celiac patients consumed approximately 7 g of gluten daily, which is about half of the average adult daily gluten intake in The Netherlands<sup>[24]</sup>. Despite this high gluten dose, no substantial histological, serological, or symptom changes were observed with placebo after 2 wk. In another study<sup>[25]</sup> in which adult CD patients consumed approximately 3.5 g/d of gluten from cracker biscuits for 2 wk, only few patients consuming gluten on placebo showed deterioration on histology, serology, and symptoms. Two other studies investigating a gluten challenge in adult patients, based on either lower gluten intake (2.5-5.0 g/d for at least 3 mo)<sup>[26]</sup> or comparable gluten intake (4 slices of white bread daily; approximately 8 g/d)<sup>[27]</sup> showed that a moderate gluten intake can be tolerated by some patients for several weeks-to-months without significant changes in symptoms<sup>[27]</sup>, serology<sup>[26]</sup> and histology<sup>[26,27]</sup>. The time to serological and mucosal relapse and recovery after gluten re-introduction and elimination, respectively, can be highly variable among adult CD patients from several weeks up to many years<sup>[27-30]</sup>. Excluding 2 out of 16 patients that may have been more sensitive to gluten from the efficacy phase may, to a small extent, have caused sample bias by select-

ing patients being less sensitive to gluten. Nevertheless, the same population of patients that entered the efficacy phase was randomly allocated to the AN-PEP or placebo arm. Also attrition bias can be excluded since all patients remained in the study. The lack of substantial clinical response to gluten observed in this study indicates that a longer gluten challenge is likely necessary to induce a significant clinical response to gluten in the majority of patients. For the same reason a longer wash-out period should be considered. Moreover, unresponsiveness to gluten of patients being diagnosed for more than 10 years ago, suggests that future studies may benefit from selecting more recently diagnosed patients.

In conclusion, AN-PEP appeared to be safe in celiac patients. More patients and gluten challenge for a longer period of time seem to be required to induce significant clinical changes and to confirm whether the tendency of AN-PEP to reduce small bowel IgA-tTG deposits is of clinical significance. These results together with previous *in vitro* evidence that AN-PEP efficiently degrades gluten under simulated gastrointestinal conditions warrant confirmation in a larger trial.

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## COMMENTS

### Background

The only currently available treatment for celiac disease consists of life-long dietary exclusion of gluten, perceived as a substantial burden particularly due to high costs, dietary restriction, reduced social activity, and increased health worries. Alternative treatment modalities that reduce the need of dieting focus on modification of dietary components, enzymatic degradation of gluten, inhibition of intestinal permeability and modulation of the immune response. Following this, a previous report showed that the gluten-degrading *Aspergillus niger*-derived prolyl endoprotease (AN-PEP) degraded toxic gluten proteins in a food matrix into non-immunogenic gluten fragments in an *in vitro* digestion model that simulates the human gastrointestinal tract.

### Research frontiers

This is the first randomised double-blind placebo-controlled pilot-study evaluating the safety and efficacy of AN-PEP in celiac disease.

### Innovations and breakthroughs

The celiac disease quality of life scores remained relatively high during 2 wk consumption of gluten and AN-PEP indicating that patients' general well-being remained high. The enzyme AN-PEP might possibly assist in digesting unintentionally ingested amounts of gluten in those who cannot tolerate gluten. However, the primary aim of the study was not met as the placebo arm did not show any deterioration (small intestinal mucosa, celiac disease associated antibodies, and quality of life) after 2 wk of gluten consumption. Although numbers were low, mucosal IgA-tTG deposits increased in 4 patients on placebo and one on AN-PEP and decreased in one patient on AN-PEP, compared to baseline values, suggesting that AN-PEP may mitigate gluten exposure.

### Applications

The lack of substantial clinical response to gluten observed in this study indicates that more patients and gluten challenge for a longer period of time seem to be required to induce significant clinical changes and to confirm whether the tendency of AN-PEP to reduce small bowel IgA-tTG deposits is of clinical significance. These results together with previous *in vitro* evidence that AN-PEP efficiently degrades gluten under simulated gastrointestinal conditions warrant

confirmation in a larger trial.

### Peer review

The aim of the present study was to examine the safety and efficacy of AN-PEP to reduce the clinical response to gluten in patients with coeliac disease. The safety study showed that AN-PEP was safe and well tolerated by patients with celiac disease. Data on time to serological and mucosal relapse and recovery after gluten re-introduction and elimination show highly variable results, varying from several weeks up to many years. Even though the primary endpoint was not met, this study is of interest and warrant support for such an approach.

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## Laparoscopic management of totally intra-thoracic stomach with chronic volvulus

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### Abstract

**AIM:** To evaluate the outcomes of patients who underwent laparoscopic repair of intra-thoracic gastric volvulus (IGV) and to assess the preoperative work-up.

**METHODS:** A retrospective review of a prospectively collected database of patient medical records identified 14 patients who underwent a laparoscopic repair of IGV. The procedure included reduction of the stomach into the abdomen, total sac excision, reinforced hiatoplasty with mesh and construction of a partial fundoplication. All perioperative data, operative details and complications were recorded. All patients had at least 6 mo of follow-up.

**RESULTS:** There were 4 male and 10 female patients. The mean age and the mean body mass index were 66 years and 28.7 kg/m<sup>2</sup>, respectively. All patients presented with epigastric discomfort and early satiety. There was no mortality, and none of the cases were converted to an open procedure. The mean operative time was 235 min, and the mean length of hospitalization was 2 d. There were no intraoperative complications. Four minor complications occurred in 3 patients including pleural

effusion, subcutaneous emphysema, dysphagia and delayed gastric emptying. All minor complications resolved spontaneously without any intervention. During the mean follow-up of 29 mo, one patient had a radiological wrap herniation without volvulus. She remains symptom free with daily medication.

**CONCLUSION:** The laparoscopic management of IGV is a safe but technically demanding procedure. The best outcomes can be achieved in centers with extensive experience in minimally invasive esophageal surgery.

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**Key words:** Laparoscopic procedure; Hiatal hernia; Stomach; Volvulus; Mesh repair

**Core tip:** Migration of the whole stomach in to the chest cavity by rotating its longitudinal or transverse axis, namely "intra-thoracic gastric volvulus", is a very rare type of giant hiatal hernias and is associated with catastrophic complications. Laparoscopic repair of this rare condition is the most technically demanding procedure among the benign foregut surgeries. With careful attention the details, such as total excision of the hernia sac, provision of an adequate esophageal length with full mobilization of the esophagus, tensionless hiatoplasty, and a floppy fundoplication, long-term success is possible

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DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5848>

### INTRODUCTION

It is difficult to identify the true incidence of hiatal hernia

because of the absence of symptoms in a large number of patients. Hiatal hernia is most commonly associated with gastro-esophageal reflux disease (GERD), and GERD affects millions of people worldwide<sup>[1]</sup>. Ninety-five percent of hiatal hernias are the small sliding type (type I) and are not associated with life threatening complications. The remaining 5% are classified as paraesophageal and mixed types (types II and III, respectively) both of which are known as giant hiatal hernias.

Landmark articles on giant hernias were published by Skinner *et al*<sup>[2]</sup> in 1962 and Hill<sup>[3]</sup> in 1973. They reported mortality rates exceeding 27% due to catastrophic complications of paraesophageal hernia (PEH) such as obstruction, strangulation, perforation and bleeding. Although still controversial, many surgeons recommend elective surgical repair even in elderly asymptomatic patients with PEH<sup>[4]</sup>.

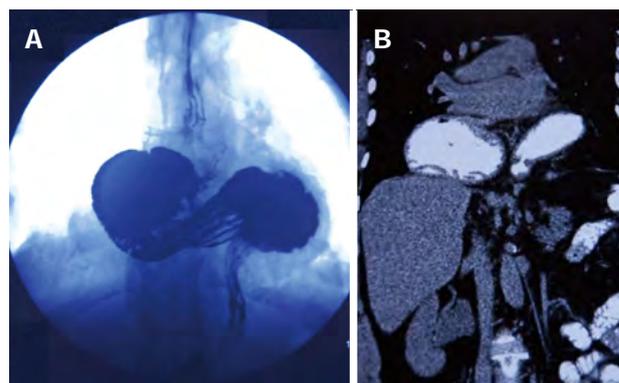
After Cuschieri *et al*<sup>[5]</sup> performed the first laparoscopic PEH repair, many surgeons reported successful results with less than 1% mortality<sup>[6,7]</sup>. All studies have shown that laparoscopic repair of giant hiatal hernias is a safe but technically demanding procedure. Because of the rarity of this disease and the lack of randomized trials comparing different surgical approaches, controversy exists regarding which surgical approach should be preferred. Choices regarding the type of surgical procedure include trans-abdominal *vs* trans-thoracic, open *vs* laparoscopic, hiatal closure with primary suture *vs* the use of meshes and whether fundoplication is necessary<sup>[8,9]</sup>.

Many previous publications addressed the management of PEH, but there is a distinct subgroup of patients who represent the end stage of all types, which occurs when the whole stomach migrates into the thorax by rotating 180 degrees around its longitudinal or transverse axis, namely “intra-thoracic gastric volvulus (IGV)”. Surgical repair of this rare disorder is most likely the most technically difficult procedure among the benign foregut diseases, even for experienced foregut surgeons. The present article focuses on this subgroup of patients who have IGV.

## MATERIALS AND METHODS

### Patient selection

The study was conducted at our anti-reflux therapy center, which is a specialized tertiary referral center for the diagnosis and treatment of GERD. A retrospective review of a prospectively collected database of patient medical records identified 14 patients who underwent laparoscopic repair for a totally intra-thoracic stomach with chronic volvulus. IGV was defined as transmigration of the whole stomach into the thorax by a 180 degree around its longitudinal or transverse axis (Figure 1). Surgical consent was obtained from all patients after detailed information was given by a senior surgeon. The preoperative evaluation included an upper gastrointestinal endoscopy, thoraco-abdominal computed tomography (CT) and a barium esophagram. We do not perform a routine



**Figure 1** Computed tomography. A: Preoperative barium swallow study shows the transmigration of the entire stomach; B: Coronal view of intra-thoracic gastric volvulus.

24-h pH study or manometry as the results do not change our treatment strategy in IGV cases. Pulmonary function testing was performed in patients with pulmonary symptoms, such as shortness of breath, to determine whether breathing problems were due to restriction of the lung by an intra-thoracic stomach or to intrinsic lung disease.

### Surgical technique

All patients were admitted on the day of the surgery and underwent a laparoscopic hiatal hernia repair procedure after an overnight fast. Patients received prophylaxis by subcutaneous low-molecular-weight heparin administered routinely during the induction of anesthesia in addition to compression stockings.

Patients were placed in the modified lithotomy position with the surgeon between the legs and the assistant on the left side. The first 10-mm optic port was placed using the open Hasson technique in the upper midline approximately one third of the way from the umbilicus to xiphoid. An additional three 10-mm and two 5-mm trocars were placed in the upper abdomen after a pneumoperitoneum was established to a pressure of 13-15 mmHg. Unlike patients who undergo an antireflux surgery, trocar placement was higher in the abdomen and was very similar to the placement in obese GERD patients we previously reported<sup>[10]</sup>.

Following liver retraction, the hiatal hernia was examined. Then the operating room table was placed in reverse Trendelenburg to allow an easier reduction. The herniated stomach was reduced into the abdomen as much as possible with atraumatic graspers in a hand-over-hand fashion. The dissection was started by dividing the gastro-hepatic ligament and exposing of the right crus. If a dominant left hepatic artery larger than 2 mm was seen, the dissection was started just above the vessel. There was no attempt to dissect the hernia sac or to find the esophagus at this stage. The hernia sac was identified at the junction of the left crus and stomach. Finding a fine areolar plane between the sac and surrounding mediastinal tissues was a landmark following the division of the hernia sac. Care was taken to identify the vagal nerves

**Table 1** Patients demographics and perioperative findings

Age, yr (range)	66.7 ± 4.7 (61-76)
Gender	
Male	4 (28.6)
Female	10 (71.4)
BMI (kg/m <sup>2</sup> )	28.7 ± 7.6
Symptoms	
Early satiety	14 (100)
Postprandial discomfort	14 (100)
Shortness of breath	2 (14.2)
Heartburn	2 (14.2)
Duration of operation (min)	234.9 ± 59.2
Day of discharge (d)	2.6 ± 1.4
Minor complication	4 (28.6)
Recurrence	1 (7.1)
Mean follow-up (mo)	29.4 ± 19

Data are expressed as absolute numbers (percentage) or mean ± SD. BMI: Body mass index.

and pleura to avoid any injury during the dissection of the mediastinum. Full division and removal of the sac was performed in all patients.

Following the sac removal, a circumferential dissection of the esophagus to the level of the inferior pulmonary veins was performed to achieve adequate intra-abdominal esophagus length. The final assessment of the esophageal length was conducted after careful dissection of the fat pad of the gastroesophageal junction (GEJ) with the operating table in a level position and with a 6-8 mmHg pneumoperitoneum. Collis gastroplasty (CP) was not routinely performed prior to fundoplication.

Once the esophageal dissection was completed, a crural repair with silk sutures was performed. Care was taken to avoid any tension over the crura during the repair, and we aimed to secure the integrity of the muscle. Reinforced hiatoplasty with prosthetic grafts was routinely performed. A U-shaped monofilament polypropylene graft (Prolene; Ethicon, Ltd.) was used for the reinforced hiatoplasty. The mesh was fixed to the diaphragm by a laparoscopic tacker. A fundoplication was performed during the procedure to avoid postoperative acid reflux. A partial posterior fundoplication, namely “Toupet fundoplication”, was the procedure of choice. We believe a better fixation of the gastric fundus is achieved with more sutures to the esophagus and crura using the Toupet procedure. Our partial fundoplication technique was standardized and reported elsewhere<sup>[11]</sup>. Briefly, the right side of the wrap was fixed to the esophagus using two silk sutures. The left part of the wrap was sutured to the anterior side of the esophagus by two or three sutures, and a single suture was used to fix the upper side of the wrap to the upper edge of the hiatus. There was no attempt to divide the short gastric vessels as the gastric fundus is too mobile due to long-term herniation.

### Postoperative period

The length of postoperative observation in the intensive care unit depended on patient co-morbidities and the

operation length. All patients were discharged on the second postoperative day unless problems occurred. Patients received liquids on the first postoperative day, after an esophagram was obtained. All patients were seen at intervals of 1 wk and 2 mo after surgery and yearly thereafter. A barium esophagram was performed during annual follow-up evaluations.

## RESULTS

Fourteen consecutive patients underwent a laparoscopic repair of IGV. There was no mortality, and none of the cases were converted to an open procedure. The duration of follow-up was 29.4 mo. There were 4 male and 10 female patients. The mean age and mean body mass index were 66.7 years and 28.7 kg/m<sup>2</sup>, respectively. All patients presented with epigastric discomfort and early satiety. Only 2 patients had additional reflux symptoms of heartburn and 2 had shortness of breath. The demographic characteristics of the patients are outlined in the Table 1.

The mean operative time was 234.9 min, and the mean length of hospitalization was 2.6 d. There were no intraoperative complications. Four minor complications occurred in three patients. One patient had pleural effusion and subcutaneous emphysema that spontaneously resolved within 2 wk. One patient had postoperative dysphagia that resolved within 6 wk without any intervention. One patient had postoperative delayed gastric emptying that began in the first postoperative week. She was treated with medical therapy and her complaints resolved within 6 mo. One patient presented with recurrent heartburn 6 mo postoperatively, and a wrap herniation was diagnosed with gastroscopy and barium swallow studies. She remains symptom free with daily proton pump inhibitor usage.

## DISCUSSION

An IGV is an uncommon entity and it occurs when the entire stomach migrates into the thorax through a giant hiatal defect by rotating around its longitudinal or transverse axis. Whether this rare condition is an extension of a PEH or an evolution of a longstanding sliding hernia is subject to controversy and is beyond the scope of this article. IGV is the end stage of all hiatal hernia types before catastrophic complications occur.

The clinical features of giant hiatal hernias are non-specific and the majority of patients are asymptomatic. Dysphagia, heartburn, postprandial discomfort and chest pain are the most common presenting symptoms<sup>[12]</sup>. Patients presenting with chest pain usually undergo a cardiac work-up and a PEH is incidentally found in chest scans. Patients with IGV are usually symptomatic, and in our study all patients presented with early satiety and postprandial discomfort.

We usually start with a gastroscopy in the preoperative work-up. In addition to detecting esophagitis and/or Barrett metaplasia, an upper gastrointestinal endoscopy

**Table 2** Laparoscopic repair of intra-thoracic gastric volvulus: Literature review

Ref.	n	Presentation	Follow-up	Mesh	Fundoplication procedure	Outcome
Inaba <i>et al</i> <sup>[15]</sup>	1	Upper abdominal pain	4 yr	PTFE	Toupet	Cure
Gökcel <i>et al</i> <sup>[16]</sup>	7	-	5 mo	PTFE	Anterior semi fundoplication	One recurrence without volvulus
Salameh <i>et al</i> <sup>[17]</sup>	1	Chest discomfort, inability to belch	1 yr	None	Nissen	Cure
Malik <i>et al</i> <sup>[18]</sup>	2	Epigastric pain, vomiting, bloating	1 yr	None	Nissen	Cure (PEG tube was placed in one patient and removed after 6 mo)
Rathore <i>et al</i> <sup>[19]</sup>	1	Chest pain, shortness of breath	1 yr	None	None	Cure
Golash <sup>[20]</sup>	1	Epigastric pain, inability to eat	6 mo	Polypropylene	Nissen+ anterior gastropexy	Cure
Iannelli <i>et al</i> <sup>[21]</sup>	1	Epigastric pain, vomiting	18 mo	-	Nissen	Cure
Krahanbuhl <i>et al</i> <sup>[22]</sup>	3	Epigastric pain, vomiting	21 mo	None	Nissen + anterior gastropexy	One recurrence with volvulus
Katkhoudan <i>et al</i> <sup>[23]</sup>	8	Epigastric pain, early satiety	16 mo	None	Nissen	One recurrence without volvulus

PEG: Percutaneous endoscopic gastrostomy.

can reveal other concomitant gastric neoplasias as the majority of patients are over 65 years old with non-specific symptoms. Unfortunately, total gastroscopy cannot be performed in most patients with IGV even under general anesthesia. Following gastroscopy, we obtain a radiographic evaluation with a barium swallow study and thoraco-abdominal CT. We think the barium swallow is very useful in identifying the presence and the type of volvulus, the location of the GEJ and in assessing the length of the esophagus. CT imaging is useful to determine possible associated organ herniation and to rule out of diaphragmatic hernia. Preoperative evaluation of patients with a pH meter and manometry is controversial. Fuller and co-workers reported 60% of patients with a giant hiatal hernia had pathological acid reflux despite the absence of typical symptoms<sup>[13]</sup>. Schieman *et al*<sup>[14]</sup> recommend routine pH meter and manometry to reveal possible concomitant reflux. We do not perform routine pH meter or manometry in our clinical practice as the results do not change our treatment strategy.

In 2013, there remains no consensus among foregut surgeons regarding the optimal surgical approach to giant hiatal hernias. The approaches include trans-abdominal *vs* trans-thoracic procedures, open *vs* laparoscopic procedures, hiatal closure with primary suture *vs* the use of meshes, fundoplication, gastropasty and total sac excision. Because of the rarity of this disease only small series and case reports exist in the literature (Table 2). As we had extensive experience in more than 1000 anti-reflux operations, a laparoscopic approach was the procedure of choice in our series. Some surgeons advocate the transthoracic approach especially in emergency cases<sup>[24]</sup>. The improved ability to separate adhesions between the hernia sac and pleura is the main advantage of trans-thoracic repair. In recent years, successful thoracoscopic repair of intrathoracic stomach has started to appear in the literature<sup>[25]</sup>. The surgeon's experience seems to be the most important consideration in choosing the procedure.

The debate over total excision of the hernia sac is the least controversial issue. Many surgeons believe total ex-

cision of the sac eliminates the tension on the GEJ and minimizes the risk of recurrence. Edey *et al*<sup>[26]</sup> addressed this issue and reported 20% early period recurrence in patients without sac excision. Although the total excision of the sac decreases the recurrence rates, some surgeons prefer to leave the distal part of the sac as a fail-safe measure to counter difficulties in dissecting nearby pleura and vagal nerves. We believe total excision is the critical step of the operation in patients with IGV, as reducing the volvulus can only be achieved by total excision. It may be very difficult when the vagus is partly adherent to the sac, especially anteriorly, and one of our patients had postoperative delayed gastric emptying after a demanding dissection. Her complaints spontaneously resolved after 6 mo, and we believe vagal injuries may not result in long-term clinical sequelae.

Short esophagus was first described in 1957<sup>[27]</sup>, and since then its pathophysiology, importance and management have remained a subject of clinical debate. Hypothetically, the inflammation of the posterior mediastinum due to the intra-thoracic stomach results in adhesion that causes esophageal shortening. The associated acid reflux can lead to chronic inflammation and fibrosis in the connective tissues that finally results in esophageal shortening. Despite the various attempts, specific criteria allowing surgeons to preoperatively identify short esophagus and to determine which patients will need a CP do not exist<sup>[28,29]</sup>. Although CP has become a more commonly used procedure in the past decade<sup>[30]</sup>, some surgeons believe that there is no need to perform CP with an adequate esophageal dissection<sup>[31]</sup>. If a 2.5-3 cm intra-abdominal esophagus can be achieved by mediastinal dissection, there is no need to perform a Collis procedure. There is a tendency to overestimate the esophageal length during a laparoscopy. The pneumoperitoneum elevates the diaphragm and misleads surgeons. Surgeons should keep in mind that these maneuvers can lead to an overestimate of intra-abdominal esophageal length. A CP was not needed in our experience. In one patient, we suspected an esophageal shortening based on a preoperative up-

per intestinal series. After mobilization of the esophagus and careful dissection of the fat pad over the GEJ, we thought we had achieved an adequate esophageal length. Unfortunately, she was the patient who presented with recurrence.

The use of prosthetic grafts for a reinforced hiato-plasty is another controversial issue in the treatment of giant hiatal hernias. The main point of controversy includes what shape, size and type of mesh should be used, and whether it should be used routinely, or in selected cases. Shamiyeh and co-workers addressed this issue by calculating the mean hiatal surface area (HSA)<sup>[32]</sup>. The authors found the average HSA was 5.84 cm<sup>2</sup> and suggested HSA can be used for the decision to use mesh. Although the use of a prosthetic mesh seems to significantly reduce the risk for recurrence<sup>[33,34]</sup>, it is not free of complications. Erosion into the gastrointestinal organs is the most feared complication when a mesh is used in the hiatus. Until recently, only a few mesh erosions were reported as single cases in the last 15 years<sup>[35]</sup>. In 2009, Stadlhuber *et al*<sup>[36]</sup> reported 28 patients with mesh complications by gathering case data from the expert esophageal surgeons. The authors suggested that the incidence mesh complications may be greater than estimated. Reinforced hiato-plasty has become routine in our early experience, even in GERD patients with small hiatal hernias. U-shaped polypropylene grafts were the preferred type of mesh. We did not observe a mesh complication in more than 700 patients. Because of the fear of mesh erosion, we used grafts more selectively after we read Stadlhuber's paper.

Adding a fundoplication procedure after the repair of the hiatus is also an issue of debate. Some surgeons recommend its selective application in patients with associated GERD<sup>[37]</sup>. Others advocate routine application because extensive dissection of the esophagus will result in GERD<sup>[38]</sup>. Nissen fundoplication is the most commonly used procedure. We routinely performed Toupet fundoplication in the present series. We can provide more fixation of the gastric fundus with more sutures. As the majority of these patients are over 65 years old, they have baseline esophageal dysmotility, and total fundoplication may result in dysphagia<sup>[39]</sup>.

As a result of negative intrathoracic pressure, there is always a tendency for the wrap to migrate back to the thorax following the repair of giant hiatal hernias. Anterior gastropexy was recommended to overcome this problem. Ponsky *et al*<sup>[40]</sup> reported a prospective study of 31 patients who underwent laparoscopic PEH repair. The authors did not observe recurrence during the 21 mo follow-up period. We believe gastropexy should not be an option in patients who have IGTV, as it may create a new axis that can lead to intra-abdominal volvulus.

In conclusion, laparoscopic management of IGTV is a safe procedure and should be the first option in the treatment algorithm. With careful attention the details, such as total excision of the hernia sac, provision of an adequate esophageal length with full mobilization of the

esophagus, tensionless hiato-plasty, and a floppy fundoplication, long-term success is possible. This procedure is most likely the most technically demanding procedure among the benign foregut diseases and requires advanced laparoscopic skills. The best outcomes can be achieved by surgeons with extensive experience, especially in laparoscopic anti-reflux surgery, as there is no learning curve for this rare condition.

## COMMENTS

### Background

Giant hiatal hernias are frequently associated with catastrophic complications such as obstruction, perforation and bleeding. Intra-thoracic gastric volvulus (IGV) is the rarest type and represents end stage of giant hiatal hernias before these complications occur.

### Research frontiers

Minimally invasive approaches for the treatment of foregut diseases are increasing worldwide. Laparoscopic management of IGV is probably most technically demanding procedure among the benign foregut diseases. The authors have focused on technically details and preoperative work-up in the management of this uncommon condition.

### Innovations and breakthroughs

Because of the rarity of IGV there is still no prospective randomized study which compares different surgical approaches and controversy exists regarding which surgical approach should be preferred such as; trans-abdominal vs trans-thoracic, open vs laparoscopic, hiatal closure with primary suture vs the use of meshes and the necessity of fundoplication. Laparoscopic approach was the procedure of choice as the authors have extensive experience in laparoscopic anti-reflux surgery. Total sac excision, tensionless hiato-plasty with mesh and Toupet fundoplication were performed in all patients without mortality and minimal morbidity.

### Applications

With careful attention the details, laparoscopic management of IGV is a safe procedure.

### Terminology

IGV is defined as transmigration of the whole stomach into the thorax by rotating 180 degrees around its longitudinal or transverse axis.

### Peer review

The authors have described their experience well in the management of this rare type of giant hiatal hernia.

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## Role of *Salmonella enterica* exposure in Chilean Crohn's disease patients

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*Salmonella enterica* (SE) and Crohn's disease (CD) and its clinical implications in Chilean patients.

**METHODS:** Ninety-four unrelated Chilean CD patients from CAREI (Active Cohort Registry of Inflammatory Bowel Disease) presenting to a single inflammatory bowel disease (IBD) unit of a University Hospital were prospectively included in this study. A complete clinical evaluation, including smoking history, was performed at the initial visit, and all the important data of clinical evolution of CD were obtained. Blood samples from these CD patients and 88 healthy sex- and age-matched control subjects were analyzed for exposure to SE and for their *NOD2/CARD15* gene status using the presence of anti-*Salmonella* lipopolysaccharide antibodies [immunoglobulin-G type (IgG)] and polymerase chain reaction (PCR), respectively. We also evaluated exposure to SE in 90 sex- and age-matched patients without CD, but with known smoking status (30 smokers, 30 non-smokers, and 30 former smokers).

**RESULTS:** CD patients comprised 54 females and 40 males, aged  $35.5 \pm 15.2$  years at diagnosis with a mean follow-up of  $9.0 \pm 6.8$  years. CD was inflammatory in 59 patients (62.7%), stricturing in 24 (25.5%) and penetrating in 15 (15.5%). Thirty cases (31.9%) had lesions in the ileum, 29 (30.8%) had ileocolonic lesions, 32 (34.0%) had colonic lesions and 23 (24.4%) had perianal disease. Sixteen CD patients (17%) were exposed to SE compared to 15 (17%) of 88 healthy control subjects ( $P = 0.8$ ). Thirty-one CD patients (32.9%) were smokers, and 7 (7.4%) were former smokers at diagnosis. In the group exposed to SE, 10 of 16 patients (62.5%) were active smokers compared to 21 of 78 patients (26.9%) in the unexposed group ( $P = 0.01$ ). On the other hand, 10 of 31 smoking patients (32%) were exposed to SE compared to 5 of 56 nonsmoking patients (9%), and one of the seven former smokers (14%) ( $P = 0.01$ ). In the group of 90 patients without CD, but whose smoking status was known, there was no differ-

### Abstract

**AIM:** To study the association between exposure to

ence in exposure to SE [3 of 30 smokers (10%), 5 of 30 non-smokers (16%), and 5 of 30 former smokers (16%);  $P = 0.6$ ]. There were no differences in disease severity between CD patients with and those without anti-SE IgG antibodies, estimated as the appearance of stricturing [2 (12.5%) *vs* 22 (28.2%);  $P = 0.2$ ] or penetrating lesions [2 (12.5%) *vs* 13 (16.6%);  $P = 1.0$ ]; or the need for immunosuppressants [11 (68.7%) *vs* 55 (70.5%);  $P = 1.0$ ], anti-tumor necrosis factor therapy [1 (6.2%) *vs* 7 (8.9%);  $P = 1.0$ ], hospitalization [13 (81.2%) *vs* 58 (74.3%);  $P = 0.7$ ], or surgery [3 (18.7%) *vs* 12 (15.3%);  $P = 0.3$ ], respectively]. No other factors were associated with SE, including *NOD2/CARD15* gene status. Seventeen CD patients (18%) had at least one mutation of the *NOD2/CARD15* gene.

**CONCLUSION:** Our study found no association between exposure to SE and CD. We observed a positive correlation between SE exposure and cigarette smoking in Chilean patients with CD, but not with disease severity.

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**Key words:** Crohn's disease; *Salmonella*; Infection; Tobacco; Smoking; Environmental factors

**Core tip:** The role and clinical implications of *Salmonella enterica* (SE) in Crohn's disease (CD) are controversial and currently unknown. We evaluated the role of exposure to SE in a cohort of Chilean patients suffering from CD. Although our study showed no association between SE exposure and CD, we observed a positive correlation between SE exposure and cigarette smoking in CD patients, but not with disease severity. These data provide evidence that more precisely defines the real role of *Salmonella* infection, an important environmental factor in CD.

Alvarez-Lobos M, Pizarro DP, Palavecino CE, Espinoza A, Sebastián VP, Alvarado JC, Ibañez P, Quintana C, Díaz O, Kalgieris AM, Bueno SM. Role of *Salmonella enterica* exposure in Chilean Crohn's disease patients. *World J Gastroenterol* 2013; 19(35): 5855-5862 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5855.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5855>

## INTRODUCTION

Crohn's disease (CD) is an immunological bowel disorder of unknown etiology, characterized by chronic relapsing inflammation of the gastrointestinal tract<sup>[1]</sup>. CD is a complex and heterogeneous disorder<sup>[2]</sup>, which is determined by, among other factors, the interaction between diverse environmental and genetic factors<sup>[3]</sup>. Studies on migrants have highlighted the importance of environmental factors in CD, and it is likely that there are important geographical differences<sup>[4,5]</sup>. Among the risk factors associated with CD are infections and smoking<sup>[6-12]</sup>, and both are major public health problems<sup>[13-15]</sup> (<http://www.ispch.cl/sites/>

[default/files/Vigilancia\\_Salmonella\\_spp\\_0](http://www.ispch.cl/sites/default/files/Vigilancia_Salmonella_spp_0)). Preliminary studies have suggested a correlation between CD and infectious gastroenteritis<sup>[16,17]</sup>. A previous report showed that, in some populations, the incidence of CD increases over time after an acute gastroenteritis episode caused by *Salmonella enterica* (SE)<sup>[18]</sup>. However, a recent report did not find an association between SE and CD<sup>[19]</sup>. Different gene-environmental interactions and gene-gene interactions have been studied in CD and are frequently related to the *NOD2/CARD15* gene<sup>[20-25]</sup>. Polymorphisms in this gene have been the most consistent and significant genetic factor implicated in CD susceptibility<sup>[26]</sup>. However, studies of environmental-environmental interaction and their implications are scarce<sup>[27,28]</sup>.

The reason for the possible association between SE and CD is unknown, but it is possible that mucosal immunity changes after SE exposure, promoting immune modification of the intestinal mucosa<sup>[29]</sup>. Thus, the intestinal mucosa may develop an enhanced inflammatory response when other factors affecting the immune response (genetic or environmental) are present. Moreover, the protein encoded by the *NOD2/CARD15* gene acts as an intracellular receptor that recognizes bacteria-derived molecules, including SE<sup>[30,31]</sup>. In addition, the clinical implications of SE in CD are unknown.

The aim of this study was to explore whether there is an association between SE exposure and CD and its implications in Chilean patients with CD.

## MATERIALS AND METHODS

### CD patients and clinical definitions

Ninety-four unrelated Chilean CD patients from CAREI (Active Cohort Registry of Inflammatory Bowel Disease) visiting a single inflammatory bowel disease (IBD) unit of a university referral center between December 2010 and June 2012 were included in this study. All CD patients gave written informed consent to participate in the study, and the local ethics committee approved the project. The study was performed in accordance with the principles stated in the declaration of Helsinki.

The diagnosis of CD was based on standard clinical, radiological, endoscopic, and histological criteria<sup>[32]</sup>. A complete clinical evaluation was performed at the initial visit, and all the important data of the clinical evolution of CD were obtained from a prospective clinical database of our unit. This evaluation included gender, age at diagnosis of CD, length of follow-up, location and behavior of CD, family history of IBD, previous appendectomy, smoking habit, oral contraceptive use, extra-intestinal manifestations of CD, and history of gastroenteritis caused by SE and/or vaccine for SE. The following characteristics were also documented: need for surgery or hospitalization for CD, use of steroids, use of immunosuppressants (azathioprine, 6-mercaptopurine and methotrexate) and use of anti-tumor necrosis factor (TNF) therapy.

Smokers were defined as patients who smoked more than seven cigarettes per week (one cigarette daily). A for-

mer smoker was defined as a patient who quit smoking at least 1 year before diagnosis. Nonsmokers were defined as patients who had never smoked or who smoke less than seven cigarettes per week<sup>[9]</sup>. The location and behavior of CD were determined according to X-ray and endoscopic findings; all patients underwent these procedures at least once at our center. The location and behavior of CD were defined according to criteria of the Montreal classification<sup>[2]</sup>; however, behaviors were registered as non-exclusive categories if intestinal stricturing occurred at a different time from the penetrating lesions; therefore, patients could belong to both categories. The severity of the disease was estimated as the appearance of stricturing or penetrating lesions or the need for immunosuppressants, anti-TNF therapy, hospitalization or surgery.

### Control patients

Eighty-eight unrelated Chilean healthy blood donors, matched by sex and age, were studied as control subjects to determine if there were any differences in the frequency of exposure to SE, independent of *NOD2/CARD15* gene status. In addition, we studied the frequency of SE exposure in a sample of patients without CD who were visiting a respiratory disease unit at our university center. These patients were differentiated by their smoking status and matched by sex and age. Thus, we studied 90 patients who were distributed as follows: 30 smokers, 30 non-smokers, and 30 former smokers. Smoking status was defined in the same way as in CD patients<sup>[9]</sup>.

### Determination of *Salmonella* exposure

For each subject, a venous blood sample was extracted to obtain serum, which was stored at -70 °C freezer until analysis. To evaluate previous exposure to *Salmonella*, serum samples were tested for the presence of anti-*Salmonella* lipopolysaccharide (LPS) antibodies [immunoglobulin-G type (IgG)] using an enzyme-linked immunosorbent assay (ELISA)<sup>[33]</sup>. The sensitivity and specificity of this methodology have been evaluated in a previous study<sup>[34]</sup>. Briefly, ELISA plates (Nalgene-Nunc®, Thermo Scientific, Waltham, MA, United States) were activated overnight at 4 °C with 500 ng of *Salmonella typhimurium* LPS (L7261-25MG SIGMA-ALDRICH, St. Louis, MO, United States) in 50 µL 0.1 mol/L bicarbonate buffer (NaHCO<sub>3</sub>, Merck, Whitehouse Station, NJ, United States) and blocked with 100 µL of PBS-BSA 3% for 1 h at room temperature. Then, 50 µL of serum diluted in PBS at 1/64, 1/128, and 1/256 was added to each well and incubated for 2 h at room temperature. Next, three washes with 200 µL of PBS-Tween 0.05% were performed. After the washes, 50 µL of an IgG conjugated with peroxidase (clone G18-145, Becton Dickinson, Franklin Lakes, NJ, United States) was added to each well (diluted 1/2000 in PBS) and incubated for 2 h at room temperature. Three washes with PBS-Tween 0.05% were then performed, and the positive reaction was developed using 3-3'-5'-tetramethyl-benzidine at a final concentration 100 µg/mL (Sigma-

Aldrich) as a colorimetric substrate. The enzymatic reaction was stopped with 2 mol/L H<sub>2</sub>SO<sub>4</sub>, and absorbance was recorded at 450 nm in an ELISA plate reader (Thermo Scientific). As a positive control, a group of subjects who had a previous history of gastroenteritis caused by SE or typhoid fever were included in this study and were used to standardize the methodology. As negative controls, a group of control subjects that had not been exposed to *Salmonella* were included, which comprised a serum of a pool of nine subjects up to 19 years old with no previous clinical history of gastroenteritis or typhoid fever. Both positive and negative controls were included in all the assays performed. Patients exposed to SE were defined as those patients with OD values higher than 2 SD over the value obtained for negative controls in the three dilutions tested in each determination. Those subjects not exposed to SE were all the other CD patients. As described before, this test allows the identification of patients that have been infected with either *Salmonella Typhimurium* or *Salmonella Enteritidis*<sup>[34]</sup>. These two serovars account for 80% of the cases of gastroenteritis caused by *Salmonella* in Chile ([http://www.ispch.cl/sites/default/files/Vigilancia\\_Salmonella\\_spp\\_0.pdf](http://www.ispch.cl/sites/default/files/Vigilancia_Salmonella_spp_0.pdf)).

### *NOD2/CARD15* genotyping

To detect *NOD2/CARD15* gene variants, genomic DNA from whole blood samples was isolated using standard molecular biology techniques. Specific sequences of exon 4 (missense mutation R702W), exon 8 (missense mutation G908R) and exon 11 (frameshift mutation L1007fsinsC) of the *Nod2/CARD15* gene were amplified by a polymerase chain reaction (PCR, using primers 5'- CCT TCA GAT CAC AGC AGC CTT C -3' and 5'- GGG ATG GAG TGG AAG TGC TTG -3' for exon 4; 5'- TCT AAG TCT GTA ATG TAA AGC CAC -3' and 5'- AGC TCC TCC CTC TTC ACC TGA -3' for exon 8, and 5'- CTG AGC CTT TGT TGA TGA GCT C -3' and 5'- ATT CTT CAA CCA CAT CCC CAT TC -3' for exon 11. PCR amplifications were performed in a MaxiGene Gradient Thermocycler (Axygen, Union City, CA, United States), using 250 ng of DNA, 1 nmol/mL of each primer, 0.2 mmol/L deoxynucleoside triphosphates, 1.5 mmol/L Magnesium Chloride and 50 U/mL of Taq DNA polymerase (Invitrogen, Carlsbad, CA, United States), using standard PCR amplification cycles. The PCR products obtained for exon 4 and exon 8 were digested with the restriction enzymes *Hpa* II and *Hba* I (New England Biolabs, Ipswich, MA, United States), and the digested PCR products were resolved by electrophoresis in 1% agarose gels containing 0.5 µg/mL ethidium bromide and visualized under a UV light transilluminator (UVP, Inc., Upland, CA, United States). PCR products obtained for exon 11 were gel purified and sequenced in an ABI 3100 automatic sequencer by Macrogen (<http://www.macrogen.com>). Frameshift mutations were analyzed for each sample, using the Vector NTI software (Invitrogen). CD patients positive for at least one of the

**Table 1 Characteristics of Crohn's disease patients *n* (%)**

Characteristic	<i>n</i> = 94
Gender	
Female	54 (57)
Age at diagnosis (yr)	
Median (range)	31 (9-80)
Early onset (< 40 yr)	61 (64.0)
Follow-up (yr)	
Median (range)	7.0 (2.0-34.0)
Smoking at diagnosis	31 (32.0)
History of gastroenteritis due to SE	5 (5.0)
Previous appendectomy	20 (21.0)
Disease localization	
Ileum	30 (31.9)
Ileocolonic	29 (30.8)
Colon	32 (31.9)
Upper gastrointestinal tract	3 (3.1)
Disease behavior	
Non-stricturing, non-penetrating	59 (62.7)
Stricturing	24 (25.5)
Penetrating	15 (15.9)
Perianal disease	23 (24.4)
Extraintestinal manifestation	49 (52.1)
Family history of IBD	14 (14.8)

SE: *Salmonella enterica*; IBD: Inflammatory bowel disease.

*NOD2/CARD15* gene polymorphisms were categorized as gene variant carriers.

### Statistical analysis

CD patients were compared to control subjects in relation to the presence of anti-SE IgG. Among CD patients, we compared those exposed to SE *vs* unexposed in relation to the most important clinical and genetic characteristics associated with CD. Categorical variables were compared using the Fisher's exact test. Continuous variables were expressed as the mean  $\pm$  SD, and compared using Student's *t* test. A *P* value of less than 0.05 was considered to indicate statistical significance. Analysis was carried out using the StatView software package (SAS Institute Inc., Cary, NC, United States).

## RESULTS

### Patient population

The study population consisted of 94 patients with CD; 54 females and 40 males. Age at diagnosis was  $35.5 \pm 15.2$  years. Mean follow-up was  $9.0 \pm 6.8$  years, with a median of 7.0 years. Behavior of CD was inflammatory in 59 patients (62.7%), stricturing in 24 patients (25.5%) and penetrating in 15 patients (15.5%). Thirty cases (31.9%) had lesions in the terminal ileum, 29 (30.8%) were ileocolonic, 32 (34.0%) had colonic lesions and 23 (24.4%) had perianal disease. Five patients (5%) reported previous gastrointestinal infection caused by *Salmonella*, and only one patient (1%) had received a vaccine for *Salmonella*. Thirty-one CD patients (32.9%) were smokers, and seven (7.4%) were former smokers at diagnosis. The clinical characteristics of CD are shown in Table 1.

**Table 2 Comparison between Crohn's disease patients exposed and those not exposed to *Salmonella enterica***

	IgG SE (+) ( <i>n</i> = 16)	IgG SE (-) ( <i>n</i> = 78)
Age at diagnosis (yr)	38.6 $\pm$ 15.6	34.9 $\pm$ 15.1
Female	6 (37.5)	48 (61.5)
Disease duration, years (yr)	11.2 $\pm$ 9.5	8.5 $\pm$ 6.1
Disease site		
Ileum	5 (31.2)	25 (32.0)
Ileocolonic	6 (37.5)	23 (29.4)
Colonic	5 (31.2)	27 (34.6)
Proximal	0 (0.0)	3 (3.8)
Perianal disease	1 (6.2)	22 (28.2)
Disease Behaviour		
Inflammatory	12 (75)	47 (60.2)
Stricturing	2 (12.5)	22 (28.2)
Penetrating	2 (12.5)	13 (16.6)
Family history of IBD	3 (18.7)	11 (14.1)
Active smokers	10 (62.5)	21 (26.9) <sup>b</sup>
Previous appendectomy	6 (37.5)	14 (17.9)
Oral contraceptive use	1 (6.2)	4 (5.1)
Extraintestinal involvement	7 (43.7)	42 (53.8)
Immunosuppressants use	11 (68.7)	55 (70.5)
Steroid use	13 (81.2)	67 (85.8)
Anti TNF therapy	1 (6.2)	7 (8.9)
Hospitalization due to IBD	13 (81.2)	58 (74.3)
Bowel Surgery	3 (18.7)	26 (33.3)
<i>NOD2/CARD15</i> Variant	5 (31.2)	12 (15.3)

Data are expressed as absolute numbers (percentage) or mean  $\pm$  SD. <sup>b</sup>*P* < 0.01 *vs* IgG *Salmonella enterica* (SE) (+). IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor.

### Exposure to SE and analysis of factors influencing exposure to SE

Sixteen CD patients (17%) were exposed to SE, as determined by the presence of anti SE-IgG in the serum. Among the 88 sex- and age-matched healthy blood donors, 15 patients (17%) were exposed to SE. There was no difference in exposure to SE between CD and control subjects (*P* = 0.8).

Comparison of the clinical characteristics between those who had been exposed to SE and those who had not, are shown in Table 2. With the exception of smoking, there were no significant differences in the clinical variables studied between the group of patients exposed to SE and those not exposed. Exposure to SE was significantly associated with cigarette smoking; in the group exposed to SE, 10 of 16 patients (62.5%) were active smokers compared to 21 of 78 patients (26.9%) in the group that was not exposed (*P* = 0.01). On the other hand, 10 of 31 smokers (32%) had exposure to SE compared with 5 of 56 nonsmokers (9%) and 1 of 7 former smokers (14%) (*P* = 0.01). No other factors were associated with exposure to SE. We also analyzed whether *NOD2/CARD15* gene variations were associated with exposure to SE, and the result was negative (*P* = 0.2, Table 2).

To assess whether increased exposure to SE was specific to CD patients who smoked, we determined the frequency of exposure to SE in smoking patients without CD. In these patients, there was no difference in exposure

to SE, defined by 3 of 30 smokers (10%) with detectable levels of anti-SE IgG compared to 5 of 30 non-smokers (16%) and 5 of 30 former smokers (16%), and the *P* value was non-significant (*P* = 0.6).

Age at diagnosis of CD, sex distribution, duration of CD, CD location, family history of IBD, and extraintestinal manifestations were similar in both groups of patients (Table 2), whereas other environmental clinical characteristics, such as use of oral contraceptives and previous appendectomy, were similar among carriers and non-carriers of anti-SE IgG (Table 2).

### CD severity according to presence of anti-SE IgG

The prevalence of several indicators of disease severity, including stricturing or penetrating lesions, the need for immunosuppressants, anti-TNF therapy, hospitalization or surgery was similar in CD patients with and those without anti-SE IgG antibodies (Table 2).

### NOD2/CARD15 genotype

Seventeen CD patients (18%) had at least one mutation of the *NOD2/CARD15* gene. All were heterozygous for these variants. The distribution of the three variants was as follows: 11 (11%) for R702W, one (1%) for G908R and five (5%) for L1007fsinsC. This frequency was higher than in control subjects, with 3 of 88 healthy blood donors (3%) displaying least 1 *NOD2/CARD15* gene mutation (*P* = 0.003).

## DISCUSSION

This study evaluated the role of *Salmonella* in CD and its interactions with genetic (*NOD2/CARD15* gene) and environmental risk factors in Chilean patients. This is a step towards unraveling the importance of environmental factors in CD and their association with other risk factors in a new population. In CD, an important part of the disease pathogenesis could be related to environmental factors and their interaction with other patient factors<sup>[1,3,11,22,27]</sup>. Our study showed no difference in the level of previous exposure to *Salmonella* between CD patients and controls. On the other hand, we observed a striking correlation between smoking and exposure to SE in CD, independent of *NOD2/CARD15* gene variants, implying a particular pattern of environment-environment interaction.

The role of microbes in CD is supported by the fact that most mouse models of IBD develop colitis only in the presence of intestinal bacteria<sup>[35]</sup>, and several human studies have shown remissions in CD patients after antibiotic therapy<sup>[36]</sup>. A growing body of evidence suggests a correlation between CD and infectious gastroenteritis<sup>[16,17]</sup> ([http://www.ispch.cl/sites/default/files/Vigilancia\\_Salmonella\\_spp\\_0.pdf](http://www.ispch.cl/sites/default/files/Vigilancia_Salmonella_spp_0.pdf)). Although a previous study implicated *Salmonella* as a risk factor for CD, in which patients with gastroenteritis caused by *Salmonella* had a higher risk for developing IBD compared to age- and sex-matched controls<sup>[18]</sup>, our study showed no difference

in previous exposure to *Salmonella* between CD patients and controls. The design of our study differs from other studies because the diagnostic criterion was based on a cross serological test to determine previous exposure to SE using the presence of anti-*Salmonella* IgG. Given the relatively low rate of history of previous typhoid fever or gastroenteritis caused by SE in our CD patient population, it is possible that independent factors of gastrointestinal infections may be more relevant in our population, including non-*NOD2/CARD15* genes, given the low frequency of mutations in this gene in our CD patients. However, our findings agree with a recent report that did not find an association between SE and CD, and this study strongly suggests that the positive associations observed in the earlier studies were the result of a detection bias<sup>[19]</sup>. The study of Jess *et al*<sup>[19]</sup> showed that the temporal risk patterns for IBD are not different following negative and positive stool tests for SE. This observation strongly suggests that increased occurrence of *Salmonella* around the time of diagnosis results from detection bias resulting from increased rates of stool testing. Moreover, the occurrence of *Salmonella* infection in patients with nonspecific gastrointestinal symptoms compatible with CD could represent a "by chance" finding and should not exclude the patients from subsequent clinical examination if gastrointestinal symptoms persist<sup>[19]</sup>.

In other autoimmune diseases, such as rheumatoid arthritis (RA), smoking is a well-established environmental risk factor<sup>[15,37,38]</sup>. In CD, smoking is also a major risk factor, and it is associated with several complications over the course of CD<sup>[9,10,39]</sup>. A meta-analysis supports the view that current smoking is associated with a significantly higher risk of CD<sup>[40]</sup>. Recent studies have reinforced the importance of smoking, suggesting that studies of risk factors for IBD should be stratified for smoking behavior, especially in cohorts of limited sample size, as in the current study<sup>[41]</sup>.

We found a significant association between *Salmonella* exposure and active smoking in a group of Chilean CD patients. These two factors may have an additive effect on the development of the disease in a subset of CD patients. To date, there is no clear biological explanation for why smoking is associated with an increased risk of CD<sup>[42]</sup>. In smokers with CD, an increased immune response against *Salmonella* could be developed, based on a greater presence of anti-*Salmonella* IgG. SE may produce a change in mucosal immunity<sup>[29]</sup>, and this change could be exacerbated in smoking patients. This could lead to tissue damage and the onset of IBD in susceptible hosts. *Salmonella* triggers an inflammatory response categorized as Th1<sup>[43]</sup>, and given that intestinal immune response in CD is classically recognized as Th1, it is conceivable that invasive bacteria, such as *Salmonella*, could trigger this abnormal intestinal immune reaction. In addition, inflammation is required by SE to colonize intestinal mucosa and compete with resident microflora<sup>[44]</sup>. In experimental studies, prolonged exposure to concentrated smoke leads to decreased expression and activity of the anti-inflam-

matory enzyme heme oxygenase-1<sup>[45]</sup>, and an increase in cell autophagy in the bowel<sup>[46]</sup>. It is possible that chronic smoking may cause a pro-inflammatory state, and *Salmonella* infection could be facilitated in smoking patients. *Salmonella* infection and tobacco consumption are a major public health problem in Chile and worldwide<sup>[9,13-15]</sup> ([http://www.bcn.cl/carpeta\\_temas/tema\\_as\\_portada.2006-09-25.0806013222/documentospdf-sobre-obesidad/VIGIA20.pdf](http://www.bcn.cl/carpeta_temas/tema_as_portada.2006-09-25.0806013222/documentospdf-sobre-obesidad/VIGIA20.pdf) and [http://www.redsalud.gov.cl/portal/docs/page/minsalcl/g\\_home/submenu\\_portada\\_2011/ens2010.pdf](http://www.redsalud.gov.cl/portal/docs/page/minsalcl/g_home/submenu_portada_2011/ens2010.pdf)), and there should be a greater emphasis on the importance of food safety and smoking avoidance.

Notably, we did not observe any association with a higher severity in patients exposed to SE compared to those not exposed, defined as the presence of stricturing or penetrating lesions or the need for immunosuppressants, anti-TNF therapy, hospitalization or surgery.

A limitation of our study is the relatively small sample population; therefore, future larger studies will be needed to confirm the relationship between smoking and infection with SE. However, the absence of this association in a control sample without CD supports the notion that this is a specific feature of CD patients.

In conclusion, our study found no association between exposure to SE and CD in a new well-defined population of Chilean CD patients. We observed a positive correlation between SE exposure and cigarette smoking in patients with CD, but not with disease severity. This research more precisely defines the real role of *Salmonella* exposure, an important environmental factor in CD, and how risk factors combine to trigger CD in a given patient.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Different environmental factors have been studied in Crohn's disease (CD). An important environmental factor that has been implicated as a possible trigger of CD is infectious gastroenteritis. There is a major controversy concerning the role of *Salmonella enterica* (SE) as a risk factor for CD. It is possible that there are important geographical differences; however, to date, there are still no data addressing the interactions between SE exposure and other environmental factors and the clinical implications of these interactions in CD.

### Research frontiers

Different gene-environment and gene-gene interactions have been studied in CD. However, environment-environment interaction studies and their clinical implications are scarce. There may be important ethnic variations. The focus of this research was to establish the real importance of an environmental factor as infectious gastroenteritis caused by SE in Chilean CD patients.

### Innovations and breakthroughs

Results of previous studies on the role of SE in CD have been controversial. The present study was designed to evaluate the role of SE in CD and its interactions with genetic and environmental risk factors in Chilean patients. The authors determined previous exposure to SE defined by the presence of anti-*Salmonella* IgG. Although their study showed no difference in previous exposure to *Salmonella* between CD patients and controls, they observed that smoking was

associated with exposure to SE in CD. If they analyze a key element, such as clinical implication of SE exposure, they did not observe a correlation between SE exposure and CD severity. This research more precisely defines the role of *Salmonella* infection, an important environmental factor, in CD and how risk factors combine to trigger CD in a given patient.

### Applications

The study results suggests that SE exposure is not associated with CD; however, the authors observed that smoking was associated with exposure to SE in Chilean CD patients, independent of other factors, implying a particular pattern of environment-environment interaction.

### Terminology

Gene-gene, gene-environment, or environment-environment interactions: In complex diseases, such as CD, there are different implications or consequences for the disease when two factors (gene and/or environmental) are present in the same patient.

### Peer review

This paper represents a considerable amount of good work, and it is a well-done and controlled study. It is a study in a well-defined population. This manuscript aimed to study the association between exposure to SE and CD. In addition, they also analyzed the involvement of cigarette smoking. They showed no association for the SE status and CD, but positive association between SE and smoking in CD.

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## Modulation of individual components of gastric motor response to duodenal glucose

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### Abstract

**AIM:** To evaluate individual components of the antro-

pyloro-duodenal (APD) motor response to graded small intestinal glucose infusions in healthy humans.

**METHODS:** APD manometry was performed in 15 healthy subjects (12 male;  $40 \pm 5$  years, body mass index  $26.5 \pm 1.6$  kg/m<sup>2</sup>) during four 20-min intraduodenal infusions of glucose at 0, 0.5, 1.0 and 1.5 kcal/min, in a randomised double-blinded fashion. Glucose solutions were infused at a rate of 1 mL/min and separated by 40-min "wash-out" period. Data are mean  $\pm$  SE. Inferential analyses are repeated measure analysis of variance with Bonferroni post-hoc testing.

**RESULTS:** At 0 kcal/min frequency of pressure waves were: antrum ( $7.5 \pm 1.8$  waves/20 min) and isolated pyloric pressure waves (IPPWs) ( $8.0 \pm 2.3$  waves/20 min) with pyloric tone ( $0.0 \pm 0.9$  mmHg). Intraduodenal glucose infusion acutely increased IPPW frequency ( $P < 0.001$ ) and pyloric tone ( $P = 0.015$ ), and decreased antral wave frequency ( $P = 0.007$ ) in a dose-dependent fashion. A threshold for stimulation was observed at 1.0 kcal/min for pyloric phasic pressure waves ( $P = 0.002$ ) and 1.5 kcal/min for pyloric tone and antral contractility.

**CONCLUSION:** There is hierarchy for the activation of gastrointestinal motor responses to duodenal glucose infusion. An increase in IPPWs is the first response observed.

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**Key words:** Glucose; Gastrointestinal motility; Pyloric; Antral; Duodenum; Manometry; Motor activity; Blood glucose

**Core tip:** Antro-pyloro-duodenal manometry was performed in 15 healthy subjects. Subjects were randomly given 20 min intraduodenal infusions of glucose at 0, 0.5, 1.0 and 1.5 kcal/min. Intraduodenal glucose infusion acutely increased isolated pyloric pressure wave

frequency and pyloric tone and decreased antral wave frequency in a dose-dependent fashion. A threshold for stimulation was observed at 1.0 kcal/min for pyloric phasic pressure waves and 1.5 kcal/min for pyloric tone and antral contractility. These data suggest that there is hierarchy for the activation of gastrointestinal motor responses to small intestinal glucose stimulation.

Deane AM, Besanko LK, Burgstad CM, Chapman MJ, Horowitz M, Fraser RJL. Modulation of individual components of gastric motor response to duodenal glucose. *World J Gastroenterol* 2013; 19(35): 5863-5869 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5863.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5863>

## INTRODUCTION

Gastric emptying of liquid nutrient is regulated at approximately 2-3 kcal/min by antro-pyloro-duodenal (APD) motor activity<sup>[1,2]</sup>. Nutrient in the small intestine stimulates receptors and initiates a feedback loop that affects motility of individual components of the APD area<sup>[3]</sup>. These motor changes include antral suppression<sup>[1]</sup>, and stimulation of phasic and tonic pyloric contractions<sup>[4,5]</sup>.

The precise APD motor response to nutrient probably depends in part on the macronutrient composition of the “meal”. Recent data suggest that the “doses” of lipid nutrient initiating these motor changes in health are much less than previously recognised<sup>[6]</sup>. Likewise small intestinal carbohydrate infusions at rates that are within normal gastric emptying rates have marked effects on the APD unit<sup>[7]</sup>. However, the specific threshold and/or hierarchy of the APD response to nutrient stimulation are unknown. The aim of this study was to assess the responses of the distal stomach to graded small intestinal nutrient stimulation in health.

## MATERIALS AND METHODS

### Subjects

Studies were performed in fifteen healthy volunteers [male:female, 12:4; age:  $40 \pm 5$  years; body mass index (BMI):  $26.5 \pm 1.6$  kg/m<sup>2</sup>]. Subjects were screened and excluded if they were diabetic, pregnant or breast feeding, had previous gastrointestinal surgery, a history of gastrointestinal disease or taking medications known to alter gastrointestinal motility. None of the subjects regularly smoked tobacco or drank more than 20 g of alcohol per day.

The protocol was approved by the research ethics committee of the Royal Adelaide Hospital, and each subject gave written informed consent prior to the commencement of the study.

### Measurement techniques

**Multi-lumen perfusion manometry:** APD motility

was assessed by a 100-cm multi-lumen perfusion manometric assembly (outer diameter 3.5 mm; Mui Scientific, Ontario, Canada). The assembly incorporated 15 pressure recording channels (side-holes spaced 1.5 cm apart), with a 4.5 cm sleeve-sensor, and an infusion port. Correct placement of the sleeve across the pylorus was determined using continuous measurement of the antro-duodenal transmucosal potential difference (TMPD) gradient<sup>[8]</sup>. The assembly was positioned so that five side holes (A1-A5) were located in the gastric antrum and seven in the proximal duodenum (Figure 1). The infusion port was located at the catheter tip to enable the delivery of enteral feed directly into the duodenum 9 cm distal to the pylorus. Thirteen manometric lumina were perfused with degassed water at a rate of 0.04 mL/min except for the sleeve perfused at a rate of 0.15 mL/min. To monitor TMPD two channels on either end of the sleeve were perfused with degassed 0.9% saline. Pressure and TMPD data were recorded on a computer using purpose written software program (Medical Measurement Systems, Enschede, The Netherlands)<sup>[8]</sup>.

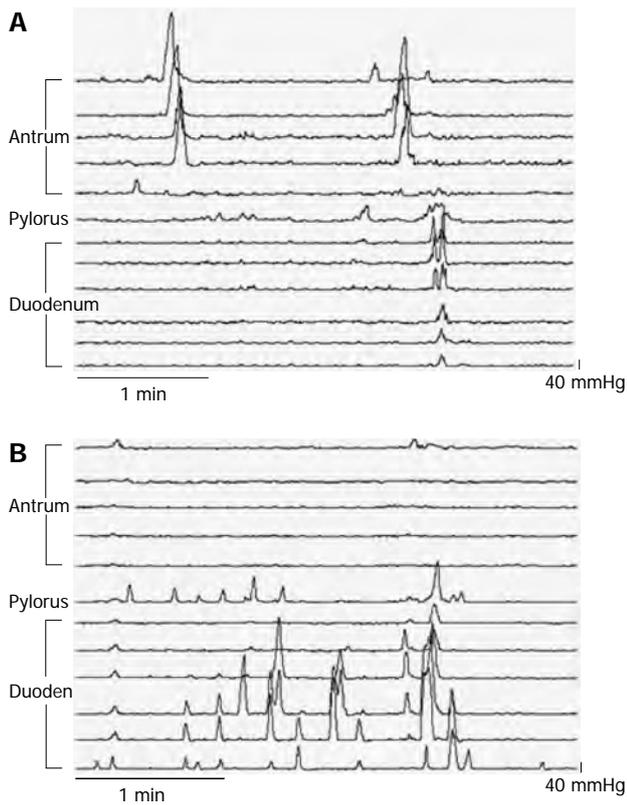
**Blood glucose concentration:** As hyperglycaemia has a major impact on gastric motility<sup>[9]</sup>, blood glucose concentrations were measured using a portable glucometer (Precision Plus, Abbott Laboratories, Bedford, United States) every 20 min throughout the study.

### Protocol

Subjects were studied in the gastrointestinal motility laboratory of the Royal Adelaide Hospital after an overnight fast. The manometric catheter was inserted into an anaesthetised nostril and passed into the stomach. The catheter passed into the duodenum assisted by spontaneous peristalsis. A cannula was inserted into an antecubital vein for blood sampling.

Each subject received intraduodenal infusions of 50% glucose solution (Pharmalab NSW Australia) diluted in water at: (1) 0.5 kcal/min; (2) 1 kcal/min; (3) 1.5 kcal; and (4) 0 kcal/min (0.9% saline only). Each solution was prepared in separate 20 mL syringes by a study investigator who was not involved in the data analysis and infused at a rate of 1 mL/min. Randomisation of glucose load was computer generated. Each syringe was then covered by the investigator preparing the syringes and labelled according to the randomisation schedule. The syringe was connected to the manometric catheter using opaque minimal volume extension tubing to ensure blinding of the research staff.

Following correct positioning of the catheter sleeve across the pylorus, a 20 min fasting period commenced. At the end of the fasting period the scheduled load was infused directly into the duodenum, *via* a volumetric syringe driver [Terumo Syringe Pump (STC-523), Medtel Australia], followed by a 40 min “washout” period of 0.9% saline (1 mL/min). A similar schedule was followed for all glucose loads. Blood samples were taken every 20 min throughout the study period to measure blood glucose



**Figure 1** Representative trace of intraluminal motility at (A) 0 kcal/min and at (B) 1.5 kcal/min. Occlusive contraction waves commence in the antrum and propagate through the duodenum. At 1.5 kcal/min isolated pyloric pressure waves occurred more frequently and a reduction in propagated antral contractility was observed.

concentrations.

### Data analysis

Manometric data were imported into Acqknowledge 3.2.7 and were analysed manually. The frequencies of APD pressure waves were determined as previously described<sup>[8]</sup>. In brief, pressure waves were included in the analysis when a rise in intraluminal pressure was greater than the minimum amplitude over the appropriate time-period and when the assembly was positioned correctly according to established TMPD criteria. Migrating motor complex (MMC) phase III activity associated pressure waves were considered to be representative of fasting motor patterns and were counted as zero for the period of phase III activity. Antral phase III MMC activity was defined as rhythmic pressure wave activity occurring at a maximum frequency (three pressure waves per minute) for at least one minute with a temporal relationship with duodenal activity. Duodenal phase III MMC was defined as a maximum frequency of 10-12 pressure waves/min for at least 2 min<sup>[8]</sup>.

A pressure wave in the antrum and pylorus was defined as a pressure rise of 10 mmHg or more from baseline and lasting between 6.1 and 20 s<sup>[10]</sup>. Isolated pyloric pressure waves (IPPWs) were defined as pressure waves at least 10 mmHg amplitude recorded only in the sleeve channel<sup>[5]</sup>. A duodenal PW was defined as a pressure rise

of 6 mmHg or more from baseline and lasting between 0.8 and 7 s<sup>[10]</sup>. Change in pyloric tone (basal pyloric pressure) was calculated as the difference in baseline pressure in the sleeve sensor from the duodenum<sup>[11]</sup> at 4-min intervals and presented as mean over 20 min.

### Statistical analysis

Data are presented as mean  $\pm$  SE. Repeated-measures analysis of variance (RM-ANOVA) were used to test for effects on pressure wave activity and pyloric tone of different caloric loads. Residuals were normally distributed and, furthermore, analyses using the equivalent non-parametric test (Friedman) remained significant. On testing there was no order effect apparent. Differences at the level of  $P < 0.05$  were considered significant and allowed post-hoc comparison between loads which were corrected according to Bonferroni adjustment.

## RESULTS

All subjects tolerated the study without adverse symptoms or effects.

### Motility

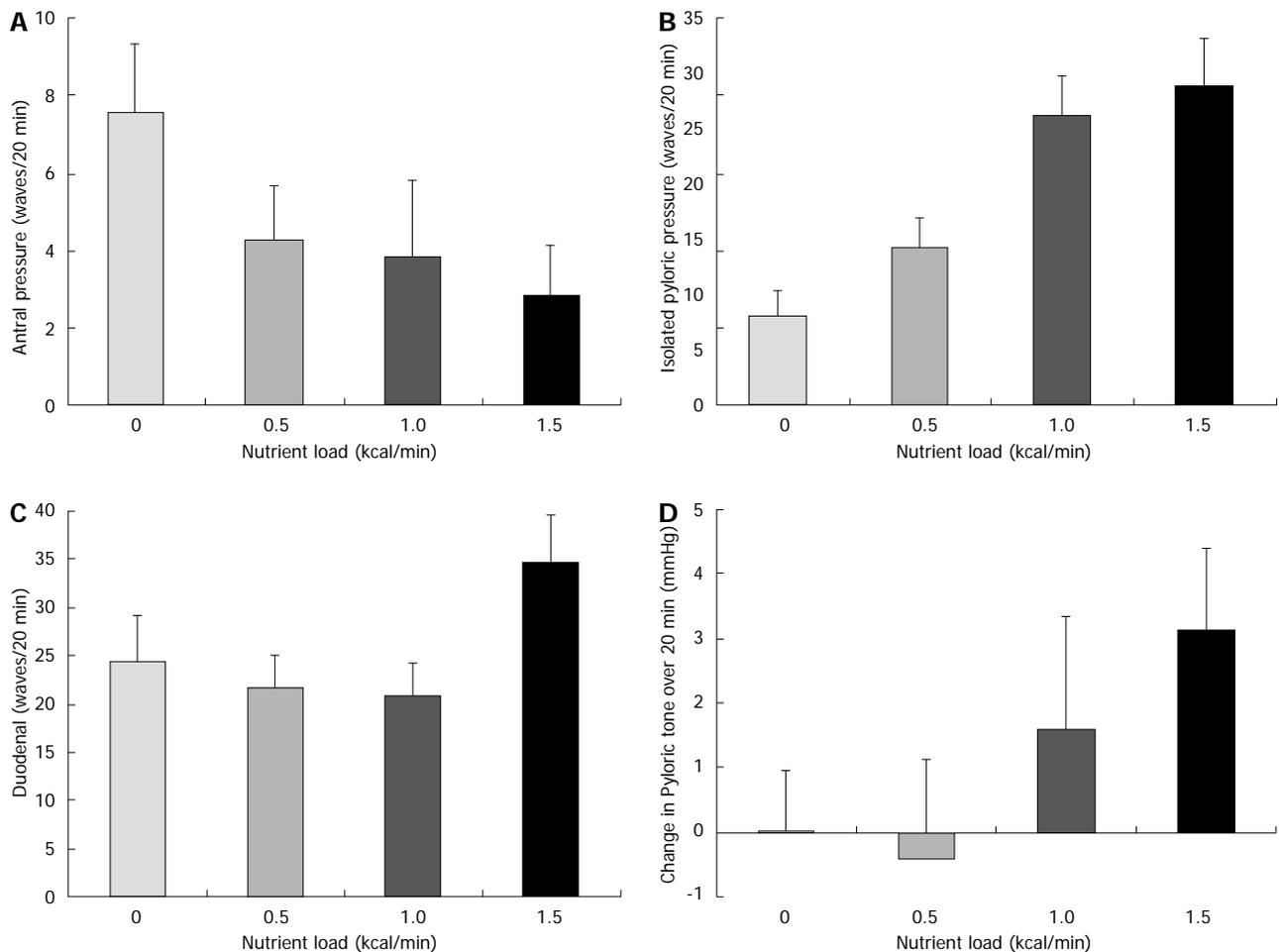
An example of a manometric trace at two different loads is shown in Figure 1.

**Antral pressure waves:** The effect of glucose loads on antral pressure wave activity is shown in Figure 2A. Increasing the caloric load had an effect on antral wave frequency ( $P = 0.007$ ) with marked attenuation of antral pressure wave activity at 1.5 kcal/min, when compared to 0 kcal/min (0 kcal/min:  $7.5 \pm 1.8$  waves/20 min *vs* 1.5 kcal:  $2.8 \pm 1.3$  waves/20 min;  $P = 0.007$ ).

**IPPWs:** The effects of glucose loads on IPPW activity are shown in Figure 2B. The frequency of IPPWs were affected by caloric load ( $P < 0.001$ ) with a substantial increase in pressure waves occurring with increasing nutrient (0 kcal/min:  $8.0 \pm 2.3$  waves/20 min *vs* 1.0 kcal:  $25.9 \pm 3.7$  waves/20 min;  $P = 0.002$ ). The increasing frequency of IPPW during glucose infusion occurred in a dose dependent fashion with 1.0 kcal/min the observed threshold to stimulate the pylorus (0 kcal/min:  $8.0 \pm 2.3$  waves/20 min *vs* 0.5 kcal/min:  $14.2 \pm 2.7$  waves/20 min;  $P = 0.294$ ; but 0.5 kcal/min  $14.2 \pm 2.7$  waves/20 min *vs* 1.0 kcal:  $25.9 \pm 3.7$  waves/20 min;  $P = 0.037$ ).

**Duodenal pressure waves:** The effects of glucose loads on duodenal wave activity are shown in Figure 2C. There was a difference between treatments over time in duodenal motor wave activity with different caloric loads ( $P = 0.012$ ). However, post-hoc testing did not reveal a difference between the individual loads (0 kcal/min:  $24.4 \pm 4.7$  waves/20 min *vs*  $34.7 \pm 4.9$  waves/20 min,  $P = 0.22$ ; and 0.5 kcal/min  $21.7 \pm 3.3$  waves/20 min *vs*  $34.7 \pm 4.9$  waves/20 min,  $P = 0.058$ ).

**Pyloric tone:** The effects of glucose loads on pyloric



**Figure 2** Antral, isolated pyloric, duodenal pressure waves and pyloric tone at differing caloric loads. A: Reduction in antral motility occurred with increasing glucose load with marked attenuation of antral pressure wave activity at 1.5 kcal/min, when compared to 0 kcal/min (0 kcal/min:  $7.5 \pm 1.8$  waves/20 min vs 1.5 kcal:  $2.8 \pm 1.3$  waves/20 min;  $P = 0.007$ ); B: The frequency of isolated pyloric pressure waves increased with increasing caloric load. The number of waves/20 min were statistically different between 0 and 1.0 kcal/min (0 kcal/min:  $8.0 \pm 2.3$  waves/20 min vs 1.0 kcal/min:  $25.9 \pm 3.7$  waves/20 min;  $P = 0.002$ ); C: The nutrient load did not effect duodenal wave frequency when comparing 0 kcal/min to 1.5 kcal/min (0 kcal/min:  $24.4 \pm 4.7$  waves/20 min vs 1.5 kcal/min:  $34.7 \pm 4.9$  waves/20 min;  $P = 0.22$ ); D: Pyloric tone increased in response to increasing caloric loads ( $P = 0.015$ ). Between 0.5 and 1.5 kcal/min the difference was significant (0.5 kcal/min:  $-0.4 \pm 1.1$  mmHg vs 1.5 kcal/min:  $3.1 \pm 1.3$  mmHg;  $P = 0.008$ ).

tone are shown in Figure 2D. There was an observed difference between treatments in pyloric tone ( $P = 0.015$ ). The difference between 0 and 1.5 kcal/min was significant prior ( $P = 0.035$ ), but not following Bonferroni adjustment ( $0.0 \pm 0.9$  vs  $3.12 \pm 1.3$ ;  $P = 0.207$ ). However, the difference remained significant between 0.5 and 1.5 kcal/min ( $-0.4 \pm 1.1$  vs  $3.1 \pm 1.3$ ;  $P = 0.008$ ).

### Blood glucose concentrations

Blood glucose concentrations were similar prior to commencing each infusion (0, 0.5, 1.0 and 1.5 kcal/min:  $6.5 \pm 0.3$  mmol/L,  $5.9 \pm 0.2$  mmol/L,  $5.8 \pm 0.3$  mmol/L vs  $5.7 \pm 0.2$  mmol/L;  $P = 0.625$ ) and at the completion of the infusion ( $5.9 \pm 0.2$ ,  $5.9 \pm 0.2$ ,  $6.4 \pm 0.3$  vs  $6.2 \pm 0.2$ ;  $P = 0.079$ ).

## DISCUSSION

The major finding of this study is that, in health, there is

a hierarchical response in the APD motor area to increasing glucose loads. The hierarchical response is graded, with initial stimulation of IPPWs and then inhibition of antral activity and an increase in pyloric tone. An increase in duodenal pressure wave frequency also occurred with a caloric load of 1.5 kcal/min.

In health, gastric emptying of nutrient liquid is regulated by the distal stomach which is affected by nutrient stimulating receptors in the small intestine<sup>[2]</sup>. Pilichiewicz *et al*<sup>[6]</sup> showed that intraduodenal infusions of as little as 0.25 kcal/min of lipid emulsion (10% intralipid) attenuated antral motility and increased pyloric phasic pressure waves. The same authors also showed that glucose at 1 kcal/min for 120 min reduced antral wave frequency but an increase in IPPWs only occurred at 4 kcal/min<sup>[7]</sup>. Pressure waves isolated to the pylorus are an integral component of the APD response to duodenal nutrient, and may be the most important mechanism to slow gastric emptying<sup>[4]</sup>. In addition, pyloric pressure waves assist

with the mixing of chyme<sup>[12]</sup> and initial stimulation of IPPWs prior to effects on other components of the APD unit should assist with trituration. Accordingly, we hypothesised that APD motor function would be affected by carbohydrate load in a hierarchical fashion and, given their substantial importance, IPPWs would occur early. It was anticipated that the magnitude of effect may be, relatively, small and the protocol was designed to detect small differences. The carbohydrate loads chosen were around 1 kcal/min<sup>[7]</sup>, which were considered physiologically relevant, the sample size was increased compared to previous studies<sup>[6,7]</sup> and the study was undertaken on a single day to minimise intrasubject variability. Lastly, the infusion periods were limited to 20 min as “adaptation” to small intestinal caloric loads has been reported during prolonged infusions<sup>[13]</sup> and if this occurred, it would have reduced the likelihood of detecting a true difference. This study shows that modulation of each component of the APD unit is hierarchical and dependent on caloric load; initially resistance to trans-pyloric flow occurs with IPPWs and, subsequently, antral propulsive force decreases. The implication of this finding is that small intestinal delivery of nutrient, even within so-called “normal” gastric emptying rates has a substantial effect on APD motor patterns.

Duodenal phasic activity is characterised by irregular motor patterns with both antegrade and retrograde pressure wave sequences<sup>[14]</sup>. These contraction wave sequences commonly propagate only over a short distance causing intermittent and bidirectional flow of chyme (to aid mixing of chyme and exposure of chyme to luminal receptors). However, more prolonged antegrade wave activity is required for aboral movement of chyme and it has been previously reported that increasing nutrient load decreases the frequency of the sequences<sup>[7,15]</sup>. In contrast, we detected a strong trend to increased duodenal activity with increasing loads. This may reflect a chance finding or the relationship between nutrient load and duodenal activity is non-linear, with initial small increments in load increasing frequency of contractions and above a certain threshold (perhaps 1.5 kcal/min) a reduction in duodenal wave frequency occurs.

The proposed mechanisms underlying modulation of the APD unit are neural and hormonal. Fone and colleagues showed that stimulation of phasic pyloric pressure waves during intraduodenal glucose at 2.4 kcal/min are mediated *via* ascending enteric nerves and ACh-stimulation of muscarinic receptors<sup>[16]</sup>. Both cholecystokinin and glucagon-like peptide-1 are secreted, albeit temporarily, in response to 1 kcal/min of intraduodenal glucose<sup>[17]</sup> and endogenous secretion of these hormones are known to have substantial effects on APD motility and, thereby, gastric emptying<sup>[18-21]</sup>.

While this study was undertaken in health, implications of these data for pathological conditions can be speculated upon. Gastric emptying is frequently slowed with healthy aging and in conditions such as diabetes and critical illness<sup>[22-24]</sup>. This slowing has been attributed

in part to “hypersensitivity” to small intestinal nutrient, which appears to be, at least in part, hormonally-mediated *via* hormones such as cholecystokinin<sup>[25]</sup>. These data in health suggest the hypothesis that the process of aging or the effects of critical illness exacerbates this hierarchical sensitivity. Further study in this area would be of interest.

The limitations of this study should be recognised. Only occlusive pressure waves are detected by manometry. Non-occlusive antral pressure waves and/or non-peristaltic flow may also have been affected<sup>[26]</sup>. However pyloric closure prevents transpyloric flow. It would be of interest to repeat this study using measurement techniques that detect non-occlusive antral pressure waves. In addition, the duodenal nutrient infusion was non-pulsatile and short term (20 min). However, the feedback mechanism is the same whether the intra-duodenal nutrient is infused in a pulsatile or non-pulsatile fashion<sup>[27]</sup>. The aim of this study was only to measure the acute response to duodenal nutrient infusion and as motor responses adapt<sup>[13]</sup>, particularly at lower loads<sup>[17]</sup>, the response during more prolonged stimulation at these loads could be different. It should also be recognized that blood glucose concentrations were not clamped and hyperglycemia, even within the physiological range, affects APD motor responses and gastric emptying<sup>[28-30]</sup>. However glucose concentrations were similar despite the differing loads, suggesting that this is unlikely to explain the adaptive response observed.

In summary, glucose loads as low as 1 kcal/min infused into the duodenum initiate APD motor responses that will slow gastric emptying. The acute APD motor response to an intra-duodenal carbohydrate load is hierarchical and “dose” dependant, with an increased frequency of IPPW, followed by a decrease in antral wave frequency.

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## COMMENTS

### Background

Emptying of liquid nutrient from the stomach is regulated by antro-pyloro-duodenal (APD) motor activity. Nutrient within the small intestine initiates a feedback loop that affects motility of the APD area. Recent data suggest that the “doses” of lipid nutrient initiating these motor changes in health are much less than previously recognised. Likewise small intestinal carbohydrate infusions at rates that are within normal gastric emptying rates have marked effects on the APD unit. However, the specific threshold and/or hierarchy of the APD response to nutrient stimulation are unknown. The aim of this study was to assess the responses of the distal stomach to graded small intestinal nutrient stimulation in health.

### Research frontiers

The specific threshold and/or hierarchy of the APD response to nutrient stimulation are unknown.

### Innovations and breakthroughs

This study shows that modulation of each component of the APD unit is hierarchical and dependent on caloric load; initially resistance to trans-pyloric flow oc-

curs with isolated pyloric pressure waves and, subsequently, antral propulsive force decreases.

### Applications

The implication of this finding is that small intestinal delivery of nutrient, even within so-called "normal" gastric emptying rates has a substantial effect on APD motor patterns.

### Terminology

APD manometry is a technique used to measure luminal occlusive contractions, which result in peristaltic flow.

### Peer review

This is a continuum of authors' previous works and tested a role of diet in the duodenum in the stimulation of the APD motor response. It's worth to publish on this journal as a brief report or communication.

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## *ITGA1* polymorphisms and haplotypes are associated with gastric cancer risk in a Korean population

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polymorphisms and haplotypes of the *ITGA1* gene and the risk of gastric cancer.

**METHODS:** The study subjects were 477 age- and sex-matched case-control pairs. Genotyping was performed for 15 single nucleotide polymorphisms (SNPs) in *ITGA1*. The associations between gastric cancer and these SNPs and haplotypes were analyzed with multivariate conditional logistic regression models. Multiple testing corrections were carried out following methodology for controlling the false discovery rate. Gene-based association tests were performed using the versatile gene-based association study (VEGAS) method.

**RESULTS:** In the codominant model, the ORs for SNPs *rs2432143* (1.517; 95%CI: 1.144-2.011) and *rs2447867* (1.258; 95%CI: 1.051-1.505) were statistically significant. In the dominant model, polymorphisms of *rs1862610* and *rs2447867* were found to be significant risk factors, with ORs of 1.337 (95%CI: 1.029-1.737) and 1.412 (95%CI: 1.061-1.881), respectively. In the recessive model, only the *rs2432143* polymorphism was significant (OR = 1.559, 95%CI: 1.150-2.114). The C-C type of *ITGA1* haplotype block 2 was a significant protective factor against gastric cancer in the both codominant model (OR = 0.602, 95%CI: 0.212-0.709,  $P = 0.021$ ) and the dominant model (OR = 0.653, 95%CI: 0.483-0.884). The *ITGA1* gene showed a significant gene-based association with gastric cancer in the VEGAS test. In the dominant model, the A-T type of *ITGA1* haplotype block 2 was a significant risk factor (OR = 1.341, 95%CI: 1.034-1.741). SNP *rs2447867* might be related to the severity of gastric epithelial injury due to inflammation and, thus, to the risk of developing gastric cancer.

**CONCLUSION:** *ITGA1* gene SNPs *rs1862610*, *rs2432143*, and *rs2447867* and the *ITGA1* haplotype block that includes SNPs *rs1862610* and *rs2432143* were significantly associated with gastric cancer.

### Abstract

**AIM:** To evaluate the association between the genetic

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**Key words:** Integrin; *ITGA1*; Gastric cancer; Polymorphism; Haplotype

**Core tip:** There are few studies addressing the role of the integrin  $\alpha 1$  subunit in the development of gastric cancer. To the best of our knowledge, this study is the first to show that *ITGA1* gene single nucleotide polymorphisms and haplotypes are associated with gastric cancer risk.

Yim DH, Zhang YW, Eom SY, Moon SI, Yun HY, Song YJ, Youn SJ, Hyun T, Park JS, Kim BS, Lee JW, Kim YD, Kim H. *ITGA1* polymorphisms and haplotypes are associated with gastric cancer risk in a Korean population. *World J Gastroenterol* 2013; 19(35): 5870-5876 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5870.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5870>

## INTRODUCTION

Gastric cancer is the second most common cancer in South Korea and represents the second leading cause of cancer death for both men and women worldwide<sup>[1,2]</sup>. Approximately one million new cases of stomach cancer are estimated to have occurred (989000 cases, 7.8% of the total), currently making it the fourth most common malignancy in the world, following cancers of the lung, breast and colo-rectum<sup>[2]</sup>. Epidemiological studies have provided evidence that a high intake of salt and nitrite-rich foods and *Helicobacter pylori* (*H. pylori*) infection are associated with a high incidence of gastric cancer in South Korea<sup>[3-7]</sup>.

The risk of developing gastric cancer is estimated to be increased 2-6 fold in patients with *H. pylori* infection<sup>[8]</sup>. The risk of gastric cancer among individuals infected with *H. pylori* is influenced by bacterial virulence. The most widely studied *H. pylori* virulence factors are the *cag* (cytotoxin-associated gene) antigens<sup>[9]</sup>. Compared to individuals infected with *cagA*-negative *H. pylori* strains, those infected with *cagA*-positive *H. pylori* strains show a higher risk of developing gastric cancer<sup>[10]</sup>. To introduce *cagA* into host cells, the *cagL* protein of *H. pylori* binds to integrins on the basolateral surface of gastric epithelial cells<sup>[11,12]</sup>.

Integrins are members of a family of heterodimeric cell-surface proteins that mediate cell-matrix and cell-cell interactions. The 18 integrin  $\alpha$ -subunits and 8  $\beta$ -subunits together form at least 25 different integrins<sup>[13]</sup>. Integrins mediate signaling events that are essential for stable cell adhesion, spreading, migration, survival, proliferation and differentiation. Several integrins, including  $\alpha 1\beta 1$ , bind to extracellular matrix proteins present in the basal membrane of mature vessels<sup>[14,15]</sup>. The tumor progression and metastasis of various cancers are associated with integrins<sup>[16,17]</sup>.

The *ITGA1* gene, located on chromosome 5q11.2,

encodes the integrin  $\alpha 1$  subunit, which is involved in the adhesion of gastric cancer cells to the peritoneum. The adhesion of integrin  $\alpha 1$ -positive gastric cancer cells to the extracellular matrix is a critical process in peritoneal dissemination<sup>[18,19]</sup>. There are few studies addressing the roles of integrins in the development of gastric cancer. An association with an increased risk of gastric cancer has only been reported for the *ITGA2* C807T polymorphism in a Chinese population<sup>[20]</sup>. As the level of integrin  $\alpha 1\beta 1$  is up-regulated in association with inflammation of the gastrointestinal tract mucosa, which is the first step in gastric carcinogenesis<sup>[21]</sup>, it is possible that the integrin  $\alpha 1$  subunit plays an important role in gastric cancer development.

The purpose of this study was to evaluate the association between the genetic polymorphisms and haplotypes of the *ITGA1* gene and the risk of gastric cancer.

## MATERIALS AND METHODS

### Study subjects

This subjects included in this study consisted of 477 newly diagnosed gastric cancer patients and an equal number of age- (within 3 years) and sex-matched controls. The diagnoses of the gastric cancer patients were all histologically confirmed at Chungbuk National University Hospital and Eulji University Hospital, which are located in a geographically central region of South Korea. Controls were selected from individuals receiving routine medical examinations in these hospitals, and individuals with a previous diagnosis of any type of cancer were excluded. Trained interviewers used a structured questionnaire including questions about demographic factors, smoking habits, alcohol consumption and dietary habits to interview all subjects who provided written informed consent. Peripheral blood samples were collected from all subjects. This study was approved by the institutional review boards of Chungbuk National University Hospital, South Korea (IRB No. 2011-09-071).

### Selection of single nucleotide polymorphisms in *ITGA1*

At the International HapMap Project website (<http://hapmap.ncbi.nlm.nih.gov/>), tag SNPs were selected using a cut-off minimum minor allele frequency in the JPT population of 0.05 and pairwise tagging ( $r^2 = 1-0.8$ ). SNPs that significantly deviated from Hardy-Weinberg equilibrium were discarded.

Genomic DNA was extracted from whole blood using the QuickGene-810 nucleic acid isolation system (Fujifilm, Tokyo, Japan) and the QuickGene DNA Whole Blood Kit (Kurabo, Osaka, Japan), in accordance with the manufacturer's instructions. DNA was stored at 4 °C until use. SNP genotyping was performed using a GoldenGate Genotyping Assay with VeraCode technology (Illumina, San Diego, CA, United States). A custom GoldenGate assay was designed for the analysis of the selected SNPs in the *ITGA1* gene. Those SNPs were then assessed for suitability for the GoldenGate genotyping platform, and

**Table 1** Characteristics of the study subjects *n* (%)

Variables	Controls ( <i>n</i> = 477)	Cases ( <i>n</i> = 477)	OR (95%CI)
Age (yr)	57.8 ± 10.2	58.7 ± 9.9	
mean ± SD			
Sex			
Males	301 (63.1)	301 (63.1)	
Females	176 (36.9)	176 (36.9)	
Smoking status			
Non-smokers	225 (47.6)	194 (41.0)	1.00 (reference)
Smokers	248 (52.4)	279 (59.0)	1.64 (0.95-2.84)
Alcohol intake status			
Non-drinkers	194 (40.7)	189 (39.6)	1.00 (reference)
Drinkers	283 (59.3)	288 (60.4)	1.18 (0.71-1.76)

the analysis was carried out on the validated SNPs. The average call rate was 99.2%. Genotyping was carried out by Macrogen (Seoul, South Korea).

### Statistical analysis

The study power was calculated using the “case-control for discrete traits” mode in the Genetic Power Calculator<sup>[22]</sup>. The following parameters were applied: risk allele frequency -0.4, alpha error -0.01, and disease prevalence -0.1%. The power of a codominant model was 0.7768 when the heterozygous OR was set to 1.5. For a dominant model, when the OR for a genotype with one or 2 risk allele(s) was taken as 2, the power was 0.8821. When a value of 2 was input for the OR for a genotype with 2 risk allele(s), the power of a recessive model was 0.8182.

Testing for deviation from the HWP was performed for each SNP in both cases and in controls using Pearson's  $\chi^2$  test. *D* values were measured using Lewontin's method for all combinations of biallelic loci<sup>[23,24]</sup>, and linkage disequilibrium blocks were structured using Haploview version 4.2 (Daly Lab at the Broad Institute Cambridge, MA, United States). Haplotype blocks were constructed and statistically compared between cases and controls with SNP Analyzer version 2.0 (ISTEC Inc., Goyang, South Korea).

Student's *t* test was used to compare continuous variables between patients and control subjects. Associations between gastric cancer and the investigated SNPs and haplotypes were estimated *via* the OR and their corresponding 95%CI derived from multivariate conditional logistic regression models, after adjusting for potential confounding factors such as age, sex, smoking history, and alcohol intake. The genotypes of major homozygotes, heterozygotes and minor homozygotes were coded as 0, 1, and 2 in the codominant model, 0, 1 and 1 in the dominant model, and 0, 0 and 1 in the recessive model, respectively. Multiple testing corrections were carried out using Benjaminin and Hochberg's methods for controlling the false discovery rate (FDR)<sup>[25]</sup>. A two-sided adjusted *P* value of < 0.05 was considered statistically significant. FDR *Q* values were calculated separately for the SNPs and haplotypes based on these numbers. Gene-based association tests were performed using the versatile gene-based association study (VEGAS) method<sup>[26]</sup>. For these statistical analyses, SAS version 9.2 (SAS Institute,

Cary, NC, United States) was employed.

## RESULTS

Patient characteristics are summarized in Table 1. No significant difference was observed between the distributions of the age, sex, and smoking and drinking habits of the cases and controls.

Table 2 lists and provides the frequencies of the 15 selected SNPs in the study subjects. None of the polymorphisms were significantly deviated from Hardy-Weinberg equilibrium. All the minor allele frequencies of the cases and controls were greater than 10%.

The haplotype linkage disequilibrium blocks and haplotype frequencies for *ITGA1* are shown in Figure 1. *D* values were measured using Lewontin's method. Four block haplotypes were constructed using Haploview version 4.2. The common haplotypes (frequency > 10%) in each block accounted for 84.2%, 99.8%, 91.6% and 99.9% for the cases and 85.7%, 99.8%, 91.2% and 99.9% for the controls.

The observed associations between the genetic polymorphisms in the *ITGA1* gene and the risk of gastric cancer are shown in Table 3. In the codominant model, the OR of 1.517 obtained for SNP *rs2432143* (95%CI: 1.144-2.011; *P* = 0.003; FDR *Q* = 0.045) was statistically significant, even after controlling the FDR, and that for *rs2447867*, of 1.258 (95%CI: 1.051-1.505; *P* = 0.012; FDR *Q* = 0.090), was marginally significant. In the dominant model, the *rs1862610* and *rs2447867* polymorphisms were not statistically significant risk factors for gastric cancer, displaying ORs of 1.337 (95%CI: 1.029-1.737; *P* = 0.029; FDR *Q* = 0.217) and 1.412 (95%CI: 1.061-1.881; *P* = 0.018; FDR *Q* = 0.217), respectively. Only the *rs2432143* polymorphism was marginally significant in the recessive model, exhibiting an OR of 1.559 (95%CI: 1.150-2.114; *P* = 0.004; FDR *Q* = 0.060).

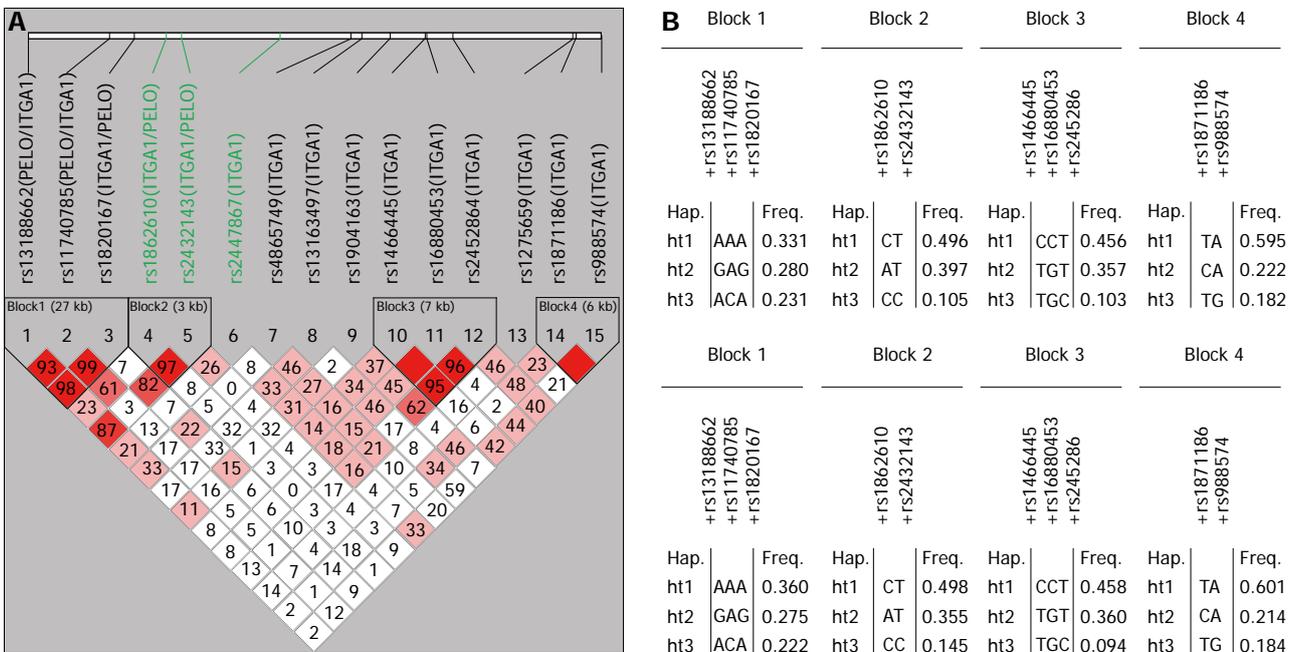
When the *P* values for the minor alleles of the codominant, dominant and recessive models were subjected to the VEGAS test, no significant gene-based associations were found. However, when the lower *P* value generated by the dominant and recessive models was input for every SNP, the value of the test statistic was 29.622, which was statistically significant (*P* = 0.037).

Four haplotype blocks were constructed using SNP Analyzer version 2.0. These blocks were evaluated for an association with the risk of gastric cancer (Table 4). The C-C type of *ITGA1* haplotype block 2 was marginally significant in the codominant model (OR = 0.602, 95%CI: 0.212-0.709; *P* = 0.021; FDR *Q* = 0.063) and was a significant protective factor against gastric cancer in the dominant model (OR = 0.653, 95%CI: 0.483-0.884; *P* = 0.006; FDR *Q* = 0.018). In the dominant model, the A-T type of *ITGA1* haplotype block 2 was a significant risk factor (OR = 1.341, 95%CI: 1.034-1.741; *P* = 0.027; FDR *Q* = 0.045). No haplotype block was found to be significant in the recessive model.

**Table 2** Frequency of *ITGA1* polymorphisms in cases and controls

SNP	Chromosomal position	Amino acid change	Genotype case/control				Case		Control	
							Frequency	HWE <sup>1</sup>	Frequency	HWE <sup>1</sup>
<i>rs13188662</i>	2686006	-	AA	AG	GG	N	0.280	0.573	0.276	0.597
<i>rs11740785</i>	2707341	-	AA	AC	CC	N	0.241	0.866	0.229	0.259
<i>rs1820167</i>	2713715	-	AA	AG	GG	N	0.435	0.806	0.420	0.904
<i>rs1862610</i>	2722239	-	CC	AC	AA	N	0.369	0.861	0.387	0.484
<i>rs2432143</i>	2725674	-	TT	TC	CC	N	0.104	0.671	0.146	0.658
<i>rs2447867</i>	2751733	C/C	CC	TC	TT	N	0.490	0.742	0.430	0.769
<i>rs4865745</i>	2770258	-	TT	TC	CC	N	0.270	0.892	0.268	0.124
<i>rs13163497</i>	2773367	-	GG	AG	AA	N	0.110	0.409	0.108	0.515
<i>rs1904163</i>	2780355	-	CC	TC	TT	N	0.298	0.196	0.272	0.698
<i>rs1466445</i>	2789486	-	CC	TC	TT	N	0.460	0.783	0.455	0.696
<i>rs16880453</i>	2789866	-	GG	GC	CC	N	0.466	0.914	0.465	0.424
<i>rs2452864</i>	2796757	-	TT	TC	CC	N	0.367	0.874	0.369	0.368
<i>rs1275659</i>	2828018	-	AA	AG	GG	N	0.257	0.185	0.278	0.864
<i>rs1871186</i>	2828974	-	TT	TC	CC	N	0.221	0.723	0.213	0.674
<i>rs988574</i>	2835169	E/G	TT	TC	CC	N	0.180	0.723	0.183	0.674
			319/309	141/155	15/9	475/473				

<sup>1</sup>P value for deviation from Hardy-Weinberg Equilibrium (HWE). SNP: Single nucleotide polymorphism.



**Figure 1** Haplotype linkage disequilibrium blocks and haplotype frequencies for *ITGA1*. A: Linkage disequilibrium (LD) blocks among *ITGA1* polymorphisms. Black squares indicate a statistically significant allelic association between a pair of single nucleotide polymorphisms, as measured by the *D* statistic; darker gray indicate higher values of *D*; B: Haplotype frequencies of *ITGA1* polymorphisms in cases (top) and controls (bottom).

**Table 3 Association between *ITGA1* polymorphisms and gastric cancer in a case-control study of a Korean population**

SNP	Chromosomal position	Codominant			Dominant			Recessive		
		OR (95%CI)	P value <sup>1</sup>	Q <sup>2</sup>	OR (95%CI)	P value <sup>1</sup>	Q <sup>2</sup>	OR (95%CI)	P value <sup>1</sup>	Q <sup>2</sup>
rs13188662	2686006	1.040 (0.840-1.281)	0.161	0.483	1.060 (0.811-1.379)	0.689	0.866	1.060 (0.660-1.690)	0.811	0.963
rs11740785	2707341	1.069 (0.869-1.313)	0.528	0.965	1.106 (0.848-1.442)	0.457	0.866	1.032 (0.630-1.692)	0.899	0.963
rs1820167	2713715	1.066 (0.884-1.286)	0.503	0.964	1.115 (0.846-1.468)	0.440	0.866	1.043 (0.751-1.447)	0.801	0.963
rs1862610	2722239	1.151 (0.965-1.372)	0.118	0.483	1.337 (1.029-1.737)	0.029	0.217	1.029 (0.740-1.429)	0.866	0.963
rs2432143	2725674	1.517 (1.144-2.011)	0.003	0.045	1.800 (0.603-5.371)	0.292	0.883	1.559 (1.150-2.114)	0.004	0.060
rs2447867	2751733	1.258 (1.051-1.505)	0.012	0.090	1.412 (1.061-1.881)	0.018	0.217	1.303 (0.966-1.756)	0.083	0.415
rs4865745	2770258	1.016 (0.829-1.246)	0.875	0.965	0.967 (0.750-1.247)	0.795	0.863	1.269 (0.759-2.122)	0.363	0.927
rs13163497	2773367	1.021 (0.768-1.357)	0.884	0.965	1.064 (0.781-1.449)	0.693	0.866	0.571 (0.167-1.952)	0.371	0.927
rs1904163	2780355	1.157 (0.943-1.420)	0.161	0.483	1.104 (0.849-1.436)	0.461	0.866	1.593 (0.984-2.577)	0.058	0.415
rs1466445	2789486	1.013 (0.845-1.213)	0.890	0.965	1.032 (0.778-1.368)	0.829	0.883	1.000 (0.736-1.358)	1.000	1.000
rs16880453	2789866	1.000 (0.832-1.201)	1.000	1.000	0.979 (0.734-1.305)	0.883	0.883	1.025 (0.752-1.398)	0.874	9.632
rs2452864	2796757	0.986 (0.816-1.191)	0.885	0.965	0.947 (0.728-1.233)	0.687	0.883	1.056 (0.728-1.532)	0.775	9.632
rs1275659	2828018	1.136 (0.919-1.404)	0.237	0.592	1.522 (0.899-2.575)	0.117	0.585	1.095 (0.841-1.427)	0.500	9.632
rs1871186	2828974	1.043 (0.841-1.293)	0.701	0.965	1.072 (0.828-1.388)	0.597	0.866	0.957 (0.533-1.716)	0.881	9.632
rs988574	2835169	0.985 (0.772-1.256)	0.901	0.965	0.927 (0.707-1.215)	0.581	0.866	1.667 (0.729-3.808)	0.225	0.843
VEGAS statistics (P)		23.986 (0.105)			16.823 (0.364)			18.732 (0.260)		

<sup>1</sup>P values for logistic analysis of three alternative models (codominant, dominant and recessive); <sup>2</sup>False discovery rate Q value. When the lower P value generated by the dominant and recessive models was applied for every single nucleotide polymorphism (SNP), the value of the versatile gene-based association study (VEGAS) statistic was 29.622 (P = 0.037).

**Table 4 Association between *ITGA1* haplotypes and gastric cancer**

Haplotypes		Codominant			Dominant			Recessive		
		OR (95%CI)	P value <sup>1</sup>	Q <sup>2</sup>	OR (95%CI)	P value <sup>1</sup>	Q <sup>2</sup>	OR (95%CI)	P value <sup>1</sup>	Q <sup>2</sup>
<i>ITGA1</i>	AAA	0.771 (0.510-1.165)	0.414	0.973	0.860 (0.666-1.112)	0.250	0.750	0.819 (0.555-1.210)	0.316	0.913
Haplotype	GAG	1.039 (0.643-1.678)	0.973	0.973	1.030 (0.799-1.328)	0.819	0.819	1.026 (0.644-1.636)	0.913	0.913
block 1	ACA	0.992 (0.559-1.760)	0.768	0.973	1.088 (0.839-1.410)	0.525	0.787	0.957 (0.544-1.683)	0.879	0.913
<i>ITGA1</i>	CT	0.982 (0.688-1.407)	0.640	0.640	1.072 (0.800-1.437)	0.641	0.641	0.911 (0.679-1.223)	0.536	0.536
Haplotype	AT	1.316 (0.686-1.407)	0.086	0.129	1.341 (1.034-1.741)	0.027	0.045	1.121 (0.784-1.603)	0.532	0.536
block 2	CC	0.602 (0.212-0.709)	0.021	0.063	0.653 (0.483-0.884)	0.006	0.018	0.661 (0.233-1.872)	0.433	0.536
<i>ITGA1</i>	CCT	1.023 (0.707-1.480)	0.677	0.794	0.934 (0.705-1.236)	0.631	0.916	0.819 (0.555-1.210)	0.316	0.913
Haplotype	TGC	0.973 (0.641-1.475)	0.314	0.794	0.986 (0.761-1.278)	0.916	0.916	1.026 (0.644-1.636)	0.913	0.913
block 3	TGT	1.418 (0.446-4.507)	0.794	0.794	1.084 (0.782-1.505)	0.627	0.916	0.957 (0.544-1.683)	0.879	0.913
<i>ITGA1</i>	TA	0.938 (0.641-1.370)	0.907	0.907	0.928 (0.658-1.310)	0.671	0.671	0.997 (0.765-1.299)	0.981	0.981
Haplotype	CA	0.983 (0.536-1.803)	0.803	0.907	1.079 (0.832-1.400)	0.567	0.671	0.952 (0.523-1.733)	0.873	0.981
block 4	TG	1.619 (0.698-3.756)	0.320	0.907	0.925 (0.708-1.209)	0.569	0.671	1.685 (0.730-3.888)	0.217	0.981

<sup>1</sup>P values for logistic analysis of three alternative models (codominant, dominant and recessive). The P value for haplotype associations were calculated using single nucleotide polymorphisms Analyzer™ 2.0 software; <sup>2</sup>False discovery rate Q value.

## DISCUSSION

The present study focused on the association of genetic polymorphisms and haplotypes of the *ITGA1* gene with gastric cancer risk. It has been suggested that the integrin  $\alpha 1$  subunit could be involved in gastric cancer carcinogenesis. Integrins on gastric epithelial cells have been reported to serve as a portal for the entry of *H. pylori cagA*<sup>[11]</sup>. Additionally, the integrin  $\alpha 1$  subunit is involved in the adhesion and dissemination of gastric cancer cells to the peritoneum<sup>[18]</sup>, and an *ITGA2* polymorphism has been reported to be associated with an increase in the risk of gastric cancer<sup>[20]</sup>. However, to our knowledge, no previous study has examined the association between *ITGA1* polymorphisms and the risk of gastric cancer.

The SNPs *rs1862610*, *rs2432143* and *rs2447867* were significantly associated with an increase in the risk of gastric cancer. After controlling the FDR, only SNP

*rs2432143* in the codominant model was statistically significant. In a gene-based association test, the *ITGA1* gene was found to be significantly associated with gastric cancer.

The C-C type of *ITGA1* haplotype block 2, which includes *rs1862610* and *rs2432143* in intron 1 of the *ITGA1* gene, was found to be a significant protective factor and the A-T type to be a risk factor for gastric cancer. This statistical significance was maintained after controlling the FDR. However, the precise molecular mechanism related to these SNPs is not clear. Based on SNP function prediction using computational methods, SNPs *rs1862610* and *rs2432143* are not predicted to be involved in any structural or functional changes in the integrin  $\alpha 1$  subunit. However, we cannot rule out the possibility that these SNPs are either associated with the stability of *ITGA1* mRNA, or in linkage disequilibrium with an as yet unknown functional polymorphism affecting the ex-

pression or function of the integrin  $\alpha 1$  subunit.

We used public databases of SNPs related to gastric cancer and assessed the potential functions of selected SNPs with SNP function prediction software. Among the 15 selected SNPs, only two were located in exons, and one was non-synonymous. The potential function was not predicted for any of these SNPs, except for *rs2447867*, which was predicted to be an exonic splicing enhancer (ESE). ESEs are clinically significant because synonymous point mutations in ESEs that were previously thought to be silent mutations can lead to exon skipping and the production of a non-functional protein. As loss of integrin  $\alpha 1\beta 1$  has been observed in some other malignancies<sup>[27]</sup>, non-functional integrin  $\alpha 1\beta 1$  could be associated with gastric cancer.

The increased expression of integrin molecules by epithelial cells during inflammation of the underlying lamina propria is probably an adaptive response to prevent extensive epithelial cell sloughing caused by inflammatory mediators. Loss of epithelial integrity due to a decrease in the function of integrin results in more severe injury of the epithelium<sup>[21]</sup>. At these sites of tissue injury, bone marrow-derived cells are recruited, and these cells can be a potential source of malignancy<sup>[28]</sup>. Because chronic infection with *H. pylori* also induces repopulation of the stomach with bone marrow-derived cells, there is a possibility that a non-functional integrin  $\alpha 1$  subunit and *H. pylori* infection would have a synergistic effect in increasing the risk of gastric cancer. The major limitation of the present study is that we did not test for the presence of antibodies against *H. pylori* and the *cagA* antigen in the sera of the case and control subjects.

The OR obtained for SNPs *rs1862610*, *rs2432143*, and *rs2447867* were all below 1.6, while the OR for the *ITGA2* C807T polymorphism in relation to gastric cancer in a Chinese population is 1.57<sup>[20]</sup>. These relatively small values can be explained by the promiscuity and redundancy of integrins: one integrin can bind several different ligands, and many different integrins can bind to the same ligand<sup>[29]</sup>. Therefore, if an integrin is not functioning, other integrins can compensate for at least some of its function.

In conclusion, the *ITGA1* gene SNPs *rs2432143* and *rs2447867* and the *ITGA1* haplotype block that includes SNP *rs2432143* are significantly associated with gastric cancer risk.

## COMMENTS

### Background

Integrins mediate signaling events that are essential for stable cell adhesion, cell spreading, migration, survival, proliferation and differentiation. Several integrins, including  $\alpha 1\beta 1$ , bind to extracellular matrix proteins present in the basal membranes of mature vessels. Tumor progression and the metastasis of various cancers are associated with integrins. The *ITGA1* gene, located on chromosome 5q11.2, encodes the integrin  $\alpha 1$  subunit, which is involved in the adhesion of gastric cancer cells to the peritoneum. Adhesion of integrin  $\alpha 1$ -positive gastric cancer cells to the extracellular matrix is a critical process in peritoneal dissemination. As integrin  $\alpha 1\beta 1$  is up-regulated during inflammation in the gastrointestinal tract mucosa, which is the first step in gastric carcinogenesis, it is possible that the integrin  $\alpha 1$  subunit plays an important role in the development of gastric cancer. It has been suggested that the integrin  $\alpha 1$  subunit could be involved in gastric cancer carcinogenesis. Integrins on gastric epithelial cells have been reported to serve as a portal for the entry of *Helicobacter pylori* (*H. pylori*) *cagA*. As integrin  $\alpha 1\beta 1$  is up-regulated during inflammation in the gastrointestinal tract mucosa, which is the first step in the gastric carcinogenesis, it is possible that the integrin  $\alpha 1$  subunit plays an important role in the development of gastric cancer.

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### Research frontiers

There are few studies addressing the role of integrins in the development of gastric cancer. An association with an increased risk of gastric cancer has only been reported previously for the *ITGA2* C807T polymorphism in a Chinese population. No earlier study has focused on the association of *ITGA1* gene single nucleotide polymorphisms (SNPs) and haplotypes with gastric cancer risk.

### Innovations and breakthroughs

To the best of the authors' knowledge, this present study is the first to suggest a significant association of the genetic polymorphisms and haplotypes of *ITGA1* gene with an increased gastric cancer risk.

### Applications

Integrins on gastric epithelial cells have been reported to serve as a portal of entry for *H. pylori* *cagA*, and loss of epithelial integrity due to a decrease in the function of integrins results in more severe injury of the epithelium. Studies are needed addressing the interaction of non-functional integrin  $\alpha 1$  subunit and *H. pylori* infection in increasing the risk of gastric cancer.

### Peer review

This paper is focused on the *ITGA1* polymorphisms and haplotypes, and gastric cancer risk in a Korean population. The results showed the SNPs *rs1862610*, *rs2432143*, and *rs2447867*, and the *ITGA1* haplotype block which includes SNPs *rs1862610* and *rs2432143* were significantly associated with gastric cancer. It is interesting.

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## Non-invasive assessment of choledocholithiasis in patients with gallstones and abnormal liver function

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### Abstract

**AIM:** To find a non-invasive strategy for detecting choledocholithiasis before cholecystectomy, with an acceptable negative rate of endoscopic retrograde cholangiopancreatography.

**METHODS:** All patients with symptomatic gallstones were included in the study. Patients with abnormal liver functions and common bile duct abnormalities on ultrasound were referred for endoscopic retrograde cholangiopancreatography. Patients with normal ultrasound

were referred to magnetic resonance cholangiopancreatography. All those who had a negative magnetic resonance or endoscopic retrograde cholangiopancreatography underwent laparoscopic cholecystectomy with intraoperative cholangiography.

**RESULTS:** Seventy-eight point five percent of patients had laparoscopic cholecystectomy directly with no further investigations. Twenty-one point five percent had abnormal liver function tests, of which 52.8% had normal ultrasound results. This strategy avoided unnecessary magnetic resonance cholangiopancreatography in 47.2% of patients with abnormal liver function tests with a negative endoscopic retrograde cholangiopancreatography rate of 10%. It also avoided unnecessary endoscopic retrograde cholangiopancreatography in 35.2% of patients with abnormal liver function.

**CONCLUSION:** This strategy reduces the cost of the routine use of magnetic resonance cholangiopancreatography, in the diagnosis and treatment of common bile duct stones before laparoscopic cholecystectomy.

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**Key words:** Magnetic resonance cholangiopancreatography; Endoscopic retrograde cholangiopancreatography; Choledocholithiasis; Liver function tests; Laparoscopic cholecystectomy; Obstructive jaundice

**Core tip:** This strategy reduces the cost of the routine use of magnetic resonance cholangiopancreatography, in the diagnosis and treatment of common bile duct stones before laparoscopic cholecystectomy.

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## INTRODUCTION

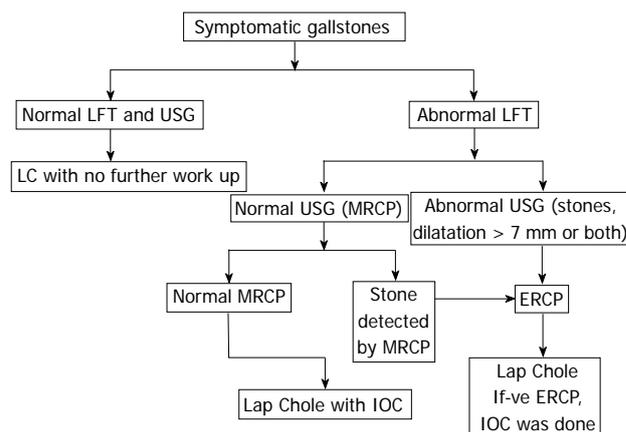
Gallstone disease is one of the most common surgical problems worldwide. Both environmental and genetic factors are known to contribute to susceptibility to the disease<sup>[1]</sup>. It has been reported that in Saudi Arabia there is an increasing incidence of gallstone disease<sup>[2]</sup>, especially in the high altitude provinces in the Asir<sup>[3]</sup> and Taif regions<sup>[4]</sup>, and similar findings have been reported for other countries that have similar environmental and nutritional factors<sup>[5,6]</sup>. Complications of gallstone disease vary from simple recurrent biliary colic to life-threatening conditions such as ascending cholangitis and pancreatitis. In addition, the disease is thought to be a risk factor for developing pancreaticobiliary cancer, particularly in patients with choledocholithiasis [common bile duct stones (CBDS)], approximately 10% of patients with symptomatic gallstones<sup>[7]</sup>.

Since symptomatic gallstone is a common indication for surgery, an accurate preoperative detection of CBDS is imperative in order to decrease operative risks and health care costs<sup>[7-9]</sup>. Better detection and treatment of CBDS before laparoscopic cholecystectomy (LC) would help deliver an appropriate, fast, and cost effective treatment<sup>[10]</sup>. Liver function tests (LFTs) and abdominal ultrasonography (USG), combined with medical history and clinical examinations are currently the standard preoperative methods used to diagnose patients with gallstones<sup>[9-14]</sup>. However, this approach is often not accurate enough to establish a firm diagnosis of CBDS<sup>[4,8,9]</sup>.

Imaging tests are routinely used to confirm a choledocholithiasis diagnosis. While abdominal USG is the most commonly used screening modality, other imaging tests used for this purpose include endoscopic and laparoscopic USG, magnetic resonance cholangiopancreatography (MRCP), intraoperative cholangiography (IOC) and endoscopic retrograde cholangiopancreatography (ERCP).

ERCP was the gold standard for diagnosing and treating CBDS; however, it is invasive, has associated morbidity and mortality, and has been shown to have a negative rate up to 75% in some studies<sup>[5]</sup>. Therefore, it was abandoned as a diagnosing method and used only for stone extraction.

MRCP is a noninvasive procedure with no associated morbidity that has become the gold standard in diagnosing CBDS; however, it should only be used when proper indications are observed<sup>[9-14]</sup> due to its high cost and limited availability<sup>[11,15,16]</sup>. Many authors have proposed its routine use<sup>[17-19]</sup> in all patients with suspected CBDS, however, in high probability patients, its cost and the need for ERCP to remove the stones makes it questionable.



**Figure 1** Algorithm followed in the management of symptomatic gallstones. LFT: Liver function test; USG: Ultrasonography; LC: laparoscopic cholecystectomy; MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography; IOC: Intraoperative cholangiography.

IOC is particularly valuable in patients with unclear anatomy and those with preoperative predictors of choledocholithiasis but negative findings on MRCP<sup>[20,21]</sup>. Many scoring indices and guidelines have been developed to determine acceptable preoperative indications for IOC, but none so far have proved satisfactory<sup>[9,22-24]</sup>. Therefore, the aim of the current study was to find a non-invasive preoperative approach, using MRCP, ERCP, and IOC, to diagnose and treat CBDS prior to performing LC.

## MATERIALS AND METHODS

We conducted a prospective study on all patients with symptomatic gallstones who presented at Al Hada Armed Forces Hospital in Taif, Saudi Arabia from April 2006 to April 2010. Patients not fit for surgery were excluded from the study. In addition, patients who presented with acute pancreatitis were excluded since we have a different protocol for them in our center. The study was approved by the local committee on human research, and all patients gave written informed consent. All patients underwent detailed preoperative evaluations consisting of clinical history, laboratory testing including LFT, (serum bilirubin, alkaline phosphatase, serum glutamic-oxaloacetic transaminase and serum glutamic pyruvic transaminase), and USG examination.

An algorithm was designed (Figure 1). Patients with normal LFT and no bile duct abnormalities were referred for LC without further work-up. Patients with abnormal LFT and USG proven CBDS and/or bile duct dilatation > 7 mm were referred for ERCP for stone removal, followed by LC. Patients with abnormal LFT and normal bile ducts (determined by USG) were referred for MRCP, and if CBDS were detected, they were referred for ERCP for stone removal, followed by LC. MRCP and ERCP negative cases underwent LC with IOC to detect false negative cases and avoid retained stones. For these patients, intraoperative discovery of CBDS would indicate

**Table 1 Demographic data n (%)**

Characteristic	Value
Total number of patients	896
Female patients	717 (80)
Male patients	179 (20)
Mean age of females (yr)	41.4 ± 21.3
Mean age of males (yr)	45.0 ± 21.6
Number of patients underwent LC without MRCP or/and ERCP	703 (78.5)
Number of patients with abnormal liver functions.	193 (21.5)
Female patients	116 (60)
Male patients	77 (40)
Mean age of females (yr)	55.6 ± 19.6
Mean age of males (yr)	60.7 ± 19.8

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; LC: Laparoscopic cholecystectomy.

**Table 2 Radiological findings in patients with abnormal liver function tests n (%)**

Findings	Patient	Stones removed by ERCP	Mean T.Bili (mg/dL)	Mean Al-P (ratio to normal)
Total	193	109	3.7	1.7
Abnormal CBD on USG (percent of total)	91 (47.2)	82 (90)	4.2	2
CBD dilatation	28 (30.8)	24 (85.7)	4.5	2
CBD stones	23 (25.3)	20 (87)	4.3	2.2
Both	40 (43.9)	38 (90)	4	1.8
Normal CBD on USG, MRCP findings (percent of total)	102 (52.8)	27 (26.5)	3.3	1.4
Normal MRCP	70 (68.6)	2 (2.9)	3.2	1.3
Stones on MRCP	32 (31.4)	25 (78.2)	3.4	1.7

MRCP: Magnetic resonance cholangiopancreatography; Al-P: Alkaline phosphatase; T.Bili: Total bilirubin; USG: Ultrasonography; CBD: Common bile duct; LFT: Liver function test; ERCP: Endoscopic retrograde cholangiopancreatography.

stone removal by ERCP in the same sitting with the LC.

**Statistical analysis**

SPSS 18.0 (SPSS, Chicago Illinois) was used for carrying out statistical analysis. Group differences were further analyzed by  $\chi^2$  and difference between means of continuous variables was tested by Student's *t* test. Multivariate logistic regression analysis was adopted to control for confounders and level of significance was determined at *P* < 0.05.

**RESULTS**

A total of 896 patients were included in the study. Table 1 shows the patient demographic information. Out of these, 703 (78.5%) patients underwent LC without any further workup. Patients who had abnormal LFTs [193 (21.5%)] were older and there were more males in this group.

Table 2 demonstrates the breakdown of all the pa-

**Table 3 Final endoscopic retrograde cholangiopancreatography findings**

Presence of stones	Patient	US abnormal	Mean T.Bili (mg/dL)	Mean Al-P (ratio to normal)
Stones	109 (56.5)	82 (90)	4.3 ± 2.1	1.9 ± 0.8
No stones	84 (43.5)	9 (10)	2.9 ± 1.3	1.3 ± 0.6
<i>P</i> value		< 0.0001	< 0.01	< 0.001
Total	193	91	3.7	1.7

Data are expressed as absolute numbers (percentage) or mean ± SD. MRCP: Magnetic resonance cholangiopancreatography; Al-P: Alkaline phosphatase; T.Bili: Total bilirubin; US: Ultrasound.

tients with abnormal LFT. CBD abnormalities were detected on USG in 91 (47.2%) patients and ERCP was used to extract stones in 90% of them. Abnormal LFT results, in which USG found dilatation but no stones were observed in 28 patients. The mean CBD diameter was 8.8 mm in these patients, with stones being extracted by ERCP in 85.7%. In 40 patients with abnormal LFT results and for whom USG detected both dilatation and stones, the mean CBD diameter was 9 mm, with stones being extracted by ERCP in 90%.

Normal bile ducts were detected by USG in 102 (52.8%) patients and ERCP was used to extract stones in 26.5% of them. IOC detected two patients with CBDS in the MRCP negative group (false negative 2.9%) and none in the ERCP negative group. There were seven patients with false positive MRCP where the ERCP did not detect any stones (false positive of 21%). This high percentage could be explained by the time between the MRCP and the ERCP that is about 2-3 d because of which the stones could have passed.

More importantly when looking at this group, 29/102 (28.4%) patients had a total bilirubin > 4 mg/dL which is counted as a high risk in recent guide lines (9); out these 17 (58.6%) patients did not have stones on IOC and ERCP was not conducted in this group of patients.

Stones were confirmed in 90% of the patients with an abnormal LFT and USG findings. ERCP detected stones in 24.5% of the patients with normal findings. This strategy avoided the use of MRCP in 47.2% of patients with abnormal LFT with a negative rate for the ERCP of 10% only. It also helped avoid unnecessary ERCP in 17 (58.6%) patients with total bilirubin > 4 mg/dL.

Statistical findings are shown in Table 3, where patients with abnormal USG, total bilirubin and alkaline phosphatase had a significant stone prediction in a univariate analysis. After controlling for confounders in multivariate Logistic regression analysis Alkaline phosphatase and USG findings were found to be significant predictors for CBDS. However, total bilirubin was found not to be a significant predictor (Table 4).

Multivariate statistical analysis found that the rise in alkaline phosphatase was a significant predictor for CBDS. It became highly significant when double the normal alkaline phosphatase value. In this case, the probability of stone detection by ERCP increased 30-fold when

**Table 4** Multivariate analysis *n* (%)

Findings	Common bile duct stones		OR <sup>1</sup>	95%CI <sup>1</sup>
	Present	Absent		
Alkaline phosphatase				
< 151 U	10 (23.3)	33 (76.7)	1	
151-225 U	37 (54.4)	31 (45.6)	3.1	7.00-9.88
226-300 U	21 (58.0)	15 (42.0)	4.5	1.23-16.49
> 300 U	41 (89.0)	5 (11.0)	29.8	7.28-121.54
Ultrasound findings				
Normal	27 (26.5)	75 (73.5)	1	
Dilatation	24 (86.0)	4 (14.0)	19.8	5.41-72.42
Stones	20 (87.0)	3 (13.0)	14.3	3.53-58.17
Both	38 (95.0)	2 (5.0)	61	13.03-285.1

<sup>1</sup>The adjusted measure of association between risk factors and common bile duct stones was expressed as the OR with 95%CI. Adjusted or crude ORs with 95%CI that did not include 1.0 were considered significant.

this enzyme level was within the normal range.

In addition, in USG findings that detected CBD dilatation and those that detected stones were both significant predictors for the presence of CBDS found by ERCP. Detection of both dilation and stones concurrently was a highly significant predictor of the presence of CBDS, which were about 60 times more likely than when USG results were normal.

## DISCUSSION

CBDS can remain hidden for years, frequently passing undetected into the duodenum. When the symptoms become apparent, the presentation will likely include obstructive jaundice or more serious complications such as acute pancreatitis or cholangitis<sup>[7,25]</sup>. Preoperative detection and intervention to remove these stones is vital<sup>[8]</sup>. An increase in bilirubin and alkaline phosphatase levels may be the only evidence of choledocholithiasis<sup>[10-12]</sup>. USG examination may confirm the presence of CBDS but cannot definitively exclude them when not detected<sup>[10]</sup>. The gold standard for treating these stones is ERCP, which has sensitivity and specificity rates both around 95%<sup>[8,11,14,26,27]</sup>. However, if the clinical, radiological, and laboratory testing indicates a low probability of choledocholithiasis, less invasive method such as MRCP should be performed first<sup>[9]</sup>.

The sensitivity of transabdominal USG is low for detecting CBDS (22%-55%), but it is better at detecting CBD dilatation (sensitivity 77%-87%). For patients with abnormal LFT which USG detects CBDS, successful diagnosis of choledocholithiasis has been reported to be above 80%. Negative CBDS detection by ERCP in such patients may be related to the passage of these stones into the duodenum before the procedure<sup>[4,28,29]</sup>.

The diameter of a normal bile duct is 3-6 mm. it increases by one mm every 10 years after the age of 60, causing mild dilatation in the elderly<sup>[30]</sup>. CBD greater than 8 mm in patients with an intact gallbladder is usually indicative of biliary obstruction<sup>[9]</sup>. Although no single factor strongly predicts choledocholithiasis in patients

with symptomatic gallstones, many studies have shown that the probability of CBDS is higher in the presence of multiple abnormal prognostic signs. Patients with such markers are considered to be at high risk, and preoperative ERCP is indicated when there are no available facilities for performing CBD laparoscopic exploration<sup>[9-13,31-34]</sup>.

In the present study, abnormal LFT results were observed in 21.5% in patients with symptomatic gallstones, a higher incidence than previously reported in the literature (15%)<sup>[35]</sup>. This discrepancy may be related to environmental (Taif is a high-altitude province), cultural or social factors<sup>[2-4,6,7]</sup>. Our facility is a tertiary hospital serving a widespread rural area.

Therefore, the routine use of MRCP as has been recommended by others for patients with abnormal LFT<sup>[17-19]</sup> is not practical or cost effective.

Among the patients involved in the study, there were 78.5% patients with normal LFT results and no CBD abnormalities detected by USG. They were therefore referred for LC without further workup, consistent with the recommendations made by the Standards of Practice Committee of the American Society for Gastrointestinal Endoscopy (SPC-ASGE)<sup>[9]</sup>. The results of our study, which concluded before the publication of the 2010 guidelines, were similar, patients with symptomatic gallstones but normal LFT and USG are considered to be at low risk for choledocholithiasis. For these patients, they recommend, as we do, cholecystectomy, without further evaluation to avoid the cost and risks of additional preoperative biliary evaluation, which are not justified by the low probability of CBDS<sup>[9]</sup>. Whether or not to perform routine IOC or laparoscopic US during LC remains an area of controversy<sup>[20,21]</sup>.

We found (52.8%) patients with abnormal LFT but normal CBD USG results, who were sent for MRCP examination. This group of patients is considered by the SPC-ASGE to be at intermediate risk of choledocholithiasis, and their guidelines recommend further evaluation with preoperative EUS or MRC or an IOC<sup>[9]</sup>, as we do. However, they do recommend that a total bilirubin higher than 4 mg/dL, should be considered as a high probability of CBDS. In our study, we found total bilirubin to be not a significant predictor on multivariate analysis. Also, in 17 (58.6%) of the patients with high total bilirubin (> 4 mg/dL) and normal USG, CBDS was not detected by IOC in the operating room. Therefore, as per their recommendation ERCP was avoided in this group in our study.

Statistical findings revealed that a rise in bilirubin level was not a significant predictor for detecting CBDS. However, this finding should be reevaluated considering the higher incidences of hepatitis, sickle cell anemia, and secondary polycythemia (related to the high altitude) in our province. Yang *et al.*<sup>[36]</sup> found that among the components of the LFTs, alkaline phosphatase was a better indicator for choledocholithiasis than bilirubin. However, the SPC-ASGE reported modestly better CBDS positive predictive values for bilirubin. They found cholestatic

liver biochemical tests, in particular alkaline phosphatase and  $\gamma$ -glutamyl transpeptidase, increases progressively with the duration and severity of biliary obstruction.

In the present study, MRCP helped avoid unnecessary ERCP in 58.6% of patients with high total bilirubin and normal CBD USG results. It was associated with a false negative rate of 2.96% and false positive rate of 21%, similar to those reported in other studies<sup>[16,37]</sup>.

Patients with abnormal LFT and USG were classified as a high risk for CBDS. By applying this algorithm, we diagnosed and treated these patients directly with ERCP and avoided the cost of MRCP and stones were extracted in 90%, with a low negative incidence of ERCP of 10%. This led to a shorter hospital stay and was even far better when some patients had the ERCP and the LC in the same sitting.

IOC has multiple advantages, as some centers use it routinely to identify the biliary anatomy and others use it for stone detection<sup>[15,16]</sup>. Its routine use is still controversial; however, in selective cases it is widely adopted. In cases where CBDS are thought of, it can be used with less cost and in the same time as the LC where it will take few minutes. However, not many general surgeons are familiar with this use, making it a less popular procedure. Therefore, its use in selective cases has been accepted. We have recommended its use in patients with negative MRCP or ERCP since these procedures have a false negative rate and discharging these patients with retained CBDS can lead to delayed acute presentation like acute pancreatitis, cholangitis and cystic duct leak<sup>[25,38]</sup>.

In conclusion, we recommend the use of this simple algorithm to stratify patients into low risk when LFT and USG are normal. These patients can go for LC with no further work-up. Patients with abnormal LFT or US are stratified as intermediate risk regardless of the total bilirubin level and should undergo MRCP as the risk of ERCP is not justified. Patients with abnormal LFT and USG are stratified as high risk and should undergo ERCP and stone extraction with LC in the same sitting if possible, as the cost of MRCP and the time needed is not justified.

## COMMENTS

### Background

Symptomatic gallstones with abnormal liver function tests are seen in higher percentages in higher altitude areas. Choledocholithiasis is the commonest cause; however, it is a challenge to diagnose and treat these cases while maintaining a low cost and minimum hospital stay. In this study the authors tried to design a simple pathway to diagnose and treat this problem without the over use of magnetic resonance cholangiopancreatography that is costly or the use of endoscopic retrograde cholangiography that has major side effects.

### Research frontiers

In the past all patients with common bile duct stones (CBDS) were subjected to endoscopic retrograde cholangiopancreatography (ERCP). This was the best way to diagnose these patients, however, there were complications like bleeding, perforation or even death with this procedure. Then magnetic resonance cholangiopancreatography (MRCP) was developed and became widely used. However, it is very costly and after the diagnosis of CBDS an ERCP was needed to remove the stone. Recently some authors have advocated the routine use of MRCP without looking in the cost or even its availability. The authors believe that it is time to reach a balance between the uses of both procedures to get

the best of both when needed.

### Innovations and breakthroughs

The authors have developed a simple pathway for the treatment of CBDS which has the least cost and requiring a minimal hospital stay. The authors have also found that the total bilirubin level does not play a part in the pathway as mentioned by the American Endoscopy Society.

### Applications

This simple pathway can be applied in any patient care facility even if it is not very advanced. The pathway does not have any special requirements.

### Peer review

The manuscript is quite well written. The methods are adequate. The results justify the conclusions drawn.

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## Erosive esophagitis associated with metabolic syndrome, impaired liver function, and dyslipidemia

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individuals with and without erosive esophagitis. Risk factors for erosive esophagitis were evaluated by multivariate logistic regression.

**RESULTS:** Erosive esophagitis was diagnosed in 507 of 5015 subjects who were individually age and sex matched to 507 esophagitis-free control subjects. In patients with erosive esophagitis, BMI, waist circumference, blood pressure, fasting plasma glucose, triglyceride levels, aspartate aminotransferase, alanine aminotransferase, the ratio of total cholesterol to HDL-C, and the ratio of low-density lipoprotein cholesterol to HDL-C were significantly higher and HDL-C was significantly lower compared to patients without erosive esophagitis (all  $P < 0.05$ ). In a multivariate analysis, central obesity (OR = 1.38; 95%CI: 1.0-1.86), hypertension (OR = 1.35; 95%CI: 1.04-1.76), hypertriglyceridemia (OR = 1.34; 95%CI: 1.02-1.76), cardiovascular risk factors as defined by a ratio of total cholesterol to HDL-C  $> 5$  (OR = 1.45; 95%CI: 1.06-1.97), and aspartate aminotransferase (OR = 1.59; 95%CI: 1.08-2.34) were significantly associated with erosive esophagitis.

**CONCLUSION:** Metabolic syndrome, impaired liver function, and a higher ratio of total cholesterol to HDL-C were associated with erosive esophagitis.

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**Key words:** Erosive esophagitis; Metabolic syndrome; Central obesity; Abnormal liver function; Dyslipidemia

### Abstract

**AIM:** To investigate whether erosive esophagitis is correlated with metabolic syndrome and its components, abnormal liver function, and lipoprotein profiles.

**METHODS:** We conducted a cross-sectional, case control study of subjects who underwent upper endoscopy during a health examination at the Health Management and Evaluation Center of a tertiary medical care facility located in Southern Taiwan. Metabolic syndrome components, body mass index (BMI), liver function, dyslipidemia, and cardiovascular risk factors, as defined by the ratio of total cholesterol to high-density lipoprotein cholesterol (HDL-C), and the ratio of low-density lipoprotein cholesterol to HDL-C were compared between

**Core tip:** A cross-sectional, case control study of subjects who underwent upper endoscopy during a health examination was conducted. Metabolic syndrome components, body mass index, liver function, and dyslipidemia were compared between individuals with and without erosive esophagitis. Risk factors for erosive esophagitis were evaluated. Erosive esophagitis was diagnosed in 507 of 5015 subjects who were individually age- and sex-matched to 507 esophagitis-free control

subjects. In addition to metabolic syndrome, we also found that abnormal liver function and predictors of future coronary heart disease were associated with erosive esophagitis.

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## INTRODUCTION

The prevalence of erosive esophagitis in Asian countries has dramatically increased during the last two decades<sup>[1-3]</sup>. The prevalence of erosive esophagitis in the Taiwanese adult population is estimated to be 10%-15%<sup>[1,4]</sup>. Although the mechanism underlying this increase in prevalence remains to be determined, several risk factors of erosive esophagitis have been identified, including male sex, hiatal hernia, smoking, alcohol consumption, and obesity<sup>[5-7]</sup>. Metabolic syndrome is a complex disorder comprising central obesity, high blood pressure (BP), hyperglycemia, hypertriglyceridemia, and a low concentration of high-density lipoprotein cholesterol (HDL-C). In addition to being associated with cardiovascular disease and diabetes mellitus, metabolic syndrome and its component elements have also been associated with various gastrointestinal diseases and abnormal liver function<sup>[7-12]</sup>. The correlation between erosive esophagitis and body mass index (BMI) is controversial<sup>[13,14]</sup>, as is the association between erosive esophagitis and hypertriglyceridemia or hyperglycemia<sup>[5,13-15]</sup>. This study was undertaken to characterize the correlation between erosive esophagitis and metabolic syndrome and its components, abnormal liver function and abnormal lipoprotein profiles - that have been used to predict coronary heart disease.

## MATERIALS AND METHODS

### Subjects

This study was designed as a cross-sectional, case control study. From January 2008 to December 2008, 5981 subjects visited the Health Management and Evaluation Center of a tertiary medical care facility located in Southern Taiwan for routine health examinations. Our center offers a variety of healthcare tests and procedures, including upper gastrointestinal endoscopy. The majority of subjects underwent a self-paid physical check-up; others were employees coming for their regular medical check-up. Most of the subjects were free of symptoms and were not chronic alcohol drinkers. Of the 5031 subjects who underwent upper gastrointestinal endoscopy, 507 were diagnosed with erosive esophagitis. The severity of erosive esophagitis was graded from A-D according

to the Los Angeles classification<sup>[16]</sup>. We matched each case subject, according to age and gender, with one control selected from the 4508 subjects with normal upper endoscopic findings. The study was approved by the institutional review board of the hospital in which the study was conducted.

### Definition of metabolic syndrome and obesity

In this study, metabolic syndrome was defined according to the modified National Cholesterol Education Program Adult Treatment Panel III for Asian populations. The waist circumference cutoff measurement was altered according to the criteria of the Bureau of Health Promotion, Department of Health, because the absolute risk of diabetes and cardiovascular disease is greater in Asians with a lesser degree of obesity<sup>[17,18]</sup>. Metabolic syndrome was diagnosed when at least three of the following criteria were found: (1) waist circumference  $\geq 90$  cm for men and  $\geq 80$  cm for women; (2) systolic BP  $\geq 130$  mmHg, diastolic BP  $\geq 85$  mmHg, or current use of antihypertensive drugs; (3) triglyceride (TG)  $\geq 150$  mg/dL; (4) HDL-C  $< 40$  mg/dL for men and  $< 50$  mg/dL for women; and (5) fasting plasma glucose  $\geq 110$  mg/dL or current use of antihyperglycemic drugs. Subjects with a BMI  $\geq 25$  kg/m<sup>2</sup> were defined as obese according to the Steering Committee of the World Health Organization Regional Office for the Western Pacific<sup>[19]</sup>. Subjects with elevated serum alanine aminotransferase (ALT) (ALT  $> 40$  U/L) or aspartate aminotransferase (AST) (AST  $> 37$  U/L) levels were considered to have abnormal liver function. Cardiovascular risk, which is determined by a ratio of total cholesterol (TC)/HDL-C  $> 5$  and correlates significantly with the risk for cardiovascular events<sup>[20]</sup>, was evaluated for its association with erosive esophagitis.

### Statistical analysis

Statistical analysis were performed using SPSS (Statistical Package for the Social Sciences) 15 software (SPSS, Chicago, IL, United States). Continuous variables are expressed as the mean  $\pm$  SD. Student's *t* test was used to compare continuous variables. Univariate analysis was performed using a  $\chi^2$  test for categorical variables. For each variable, the OR and 95%CI were calculated. A two-tailed *P* value of  $< 0.05$  was considered statistically significant. Multivariate analysis in the logistic regression model was conducted to examine the associations between erosive esophagitis and different risk factors.

## RESULTS

### Prevalence of erosive esophagitis

Of the 5031 subjects who underwent upper gastrointestinal endoscopy, 16 were excluded from the analysis because of prior gastric surgery, gastric cancer, or peptic ulcer. Erosive esophagitis was diagnosed in 507 of 5015 subjects. The mean age of subjects with erosive esophagitis was  $51.2 \pm 11.2$  years, and 82.6% were male.

**Table 1 Comparison between subjects with and without erosive esophagitis**

	With erosive esophagitis (n = 507)	Without erosive esophagitis (n = 507)	P value
Age (yr, mean ± SD)	51.2 ± 11.2	51.2 ± 11.2	-
Sex (M/F)	419/88	419/88	-
BMI (kg/m <sup>2</sup> )	25.6 ± 3.6	24.9 ± 3.4	< 0.001
Waist circumference (cm)	87.3 ± 12.9	84.8 ± 11.9	< 0.001
Systolic BP (mmHg)	132.3 ± 18.1	129.2 ± 17.7	0.003
Diastolic BP (mmHg)	87.2 ± 11.7	84.2 ± 10.8	< 0.001
Fasting plasma glucose (mg/dL)	105.9 ± 36.5	101 ± 29	0.009
Triglycerides (mg/dL)	165 ± 107.4	137.1 ± 99.5	< 0.001
HDL-C (mg/dL)	50.4 ± 15	52.2 ± 14.5	0.025
TC/HDL-C	4.27 ± 1.26	4.04 ± 1.13	0.001
LDL-C/HDL-C	2.56 ± 0.96	2.46 ± 0.87	0.049
AST (U/L)	31 ± 28.7	27.3 ± 13.4	0.004
ALT (U/L)	37.1 ± 26.4	31.2 ± 21.6	0.005

The P values are based on Student's *t* test. M: Male; F: Female; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BP: Blood pressure; BMI: Body mass index; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol.

### Characteristics of subjects with and without erosive esophagitis

The subject characteristics are summarized in Table 1. When compared with age- and sex-matched controls, BMI, waist circumference, systolic and diastolic BP, fasting plasma glucose, triglyceride levels, AST/glutamate-oxaloacetate transaminase (GOT) and ALT/glutamate-pyruvate transaminase (GPT) levels, the ratio of TC to HDL-C, and the ratio of low-density-lipoprotein cholesterol to HDL-C (LDL-C to HDL-C) were significantly higher and HDL-C was significantly lower in subjects with erosive esophagitis (all *P* < 0.05).

### Univariate and multivariate analyses of the associations between erosive esophagitis and risk factors

The results from the univariate and multivariate logistic regression analyses are shown in Table 2. BMI  $\geq$  25 kg/m<sup>2</sup> (OR = 1.72; 95%CI: 1.10-1.80), central obesity (OR = 1.60; 95%CI: 1.20-2.14), hypertension (OR = 1.43; 95%CI: 1.11-1.86), hyperglycemia (OR = 1.39; 95%CI: 1.07-1.80), hypertriglyceridemia (OR = 1.50; 95%CI: 1.15-1.95), and cardiovascular disease risk factors of TC/HDL-C > 5 (OR = 1.57; 95%CI: 1.17-2.12), AST > 37 U/L (OR = 1.67; 95%CI: 1.14-2.45), and ALT > 40 U/L (OR = 1.40; 95%CI: 1.04-1.90) were significantly associated with erosive esophagitis, according to the univariate analyses. Furthermore, the multivariate logistic regression analysis confirmed the associations of central obesity, hypertension, hypertriglyceridemia, the ratio of TC/HDL-C > 5, and high AST levels with erosive esophagitis (all *P* < 0.05).

### Association of erosive esophagitis with metabolic syndrome

Table 3 shows that the presence of metabolic syndrome

( $\geq$  3 metabolic criteria) was associated with a higher probability of erosive esophagitis than the presence of < 3 metabolic criteria (OR = 1.475; 95%CI: 1.149-1.895). The prevalence of metabolic syndrome was higher in subjects with erosive esophagitis than in those without (47.1% *vs* 37.7%, respectively; *P* < 0.005).

## DISCUSSION

In this study, erosive esophagitis was identified in 10.1% of subjects who underwent routine health examinations. Central obesity, hypertension, hypertriglyceridemia, a high TC/HDL-C ratio (TC/HDL-C > 5), and AST > 37 U/L were significantly associated with erosive esophagitis. Previously, Chua *et al*<sup>[13]</sup> showed that an increase in BMI was related to an increase in erosive esophagitis, but Chung *et al*<sup>[14]</sup> did not find a significant association between BMI and erosive esophagitis. Our study showed that central obesity, but not BMI, was an independent risk factor for erosive esophagitis.

A possible reason for this finding is that BMI is not a good indicator of the percentage of body fat among Asian populations<sup>[21]</sup>. Only the visceral component of abdominal fat increases the risk for erosive esophagitis because visceral adipose tissue is strongly associated with elevated serum levels of proinflammatory adipokines, which may play a role in the development of erosive esophagitis<sup>[7,14,22]</sup>. In addition, central obesity may increase intra-abdominal pressure and decrease lower esophageal sphincter pressure, resulting in esophageal sphincter relaxation with acid reflux, which may lead to esophagitis<sup>[22-24]</sup>.

Studies have also shown that hypertriglyceridemia is associated with erosive esophagitis<sup>[13-15]</sup>, but contrasting results have also been reported<sup>[5]</sup>. The present study shows that hypertriglyceridemia is a potential risk factor for erosive esophagitis. Although the underlying mechanisms still need to be fully characterized, high dietary fat intake and delay in gastric emptying may increase the risk of erosive esophagitis<sup>[25,26]</sup>.

The association of hyperglycemia and erosive esophagitis is controversial. Moki *et al*<sup>[5]</sup> demonstrated a positive relationship between erosive esophagitis and hyperglycemia. However, the majority of studies have found that hyperglycemia is not associated with erosive esophagitis<sup>[13-15,27]</sup>. Our results also indicate that hyperglycemia is not associated with erosive esophagitis. Gastric emptying can be delayed by diabetic autonomic neuropathy, which may promote erosive esophagitis. However, most individuals in our study population were in generally good health, without diabetic autonomic neuropathy, which may explain our finding that hyperglycemia is not associated with erosive esophagitis. The association of hypertension and erosive esophagitis is also controversial. Gudlaugsdottir *et al*<sup>[28]</sup> suggested that erosive esophagitis was associated with hypertension, but Wu *et al*<sup>[29]</sup> failed to establish a significant relationship between hypertension and erosive esophagitis. Our study showed that hyperten-

**Table 2** Logistic regression analysis of risk factors for erosive esophagitis

	Univariate analysis OR (95%CI)	P value	Multivariate analysis OR (95%CI)	P value
Obesity <sup>1</sup>	1.72 (1.10-1.80)	< 0.05		0.606
Central obesity <sup>2</sup>	1.60 (1.20-2.14)	< 0.05	1.38 (1.00-1.86)	< 0.05
Hypertension	1.43 (1.11-1.86)	< 0.05	1.35 (1.04-1.76)	< 0.05
Hyperglycemia	1.39 (1.07-1.80)	< 0.05		0.143
Hypertriglyceridemia	1.50 (1.15-1.95)	< 0.05	1.34 (1.02-1.76)	< 0.05
Low HDL-C	1.24 (0.91-1.67)	0.192	-	-
TC/HDL-C > 5	1.57 (1.17-2.12)	< 0.05	1.45 (1.06-1.97)	< 0.05
AST > 37 (U/L)	1.67 (1.14-2.45)	< 0.05	1.59 (1.08-2.34)	< 0.05
ALT > 40 (U/L)	1.40 (1.04-1.90)	< 0.05		0.986

<sup>1</sup>Defined as body mass index  $\geq 25$  kg/m<sup>2</sup>; <sup>2</sup>Defined as waist circumference  $\geq 90$  cm for men and  $\geq 80$  cm for women. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL-C: High-density lipoprotein cholesterol; TC: Total cholesterol.

**Table 3** Components of metabolic syndrome associated with erosive esophagitis *n* (%)

Metabolic factors	Erosive esophagitis ( <i>n</i> = 507)	Matched normal control ( <i>n</i> = 507)	OR (95%CI)	P value
Number of criteria				
$\geq 1$ criterion	473 (93.3)	447 (88.2)	1.867 (1.203-2.899)	0.007
$\geq 2$ criteria	391 (77.1)	335 (66.1)	1.731 (1.312-2.283)	< 0.001
$\geq 3$ criteria	239 (47.1)	191 (37.7)	1.475 (1.149-1.895)	0.003
$\geq 4$ criteria	112 (22.1)	64 (12.6)	1.963 (1.403-2.746)	< 0.001
5 criteria	28 (5.5)	20 (3.9)	1.423 (0.791-2.561)	0.301

sion is an independent risk factor for erosive esophagitis.

Several studies have reported that abnormal liver function is related to metabolic syndrome<sup>[10-12]</sup>. In addition to metabolic syndrome, high BMI, central obesity, and hyperlipidemia are associated with hepatic steatosis<sup>[30,31]</sup>. The severity of hepatic steatosis is significantly correlated with the results of hepatic enzyme tests<sup>[32]</sup>. Several studies have reported an association between gastroesophageal reflux disease (GERD) and chronic liver disease<sup>[33-36]</sup>. Suzuki *et al*<sup>[36]</sup> showed that more than 30% of patients with chronic liver disease had GERD. Ueda *et al*<sup>[34]</sup> showed a relatively higher incidence of GERD in patients with alcoholic liver disease. We found that abnormal liver function (higher AST level) was a risk factor for erosive esophagitis. Although the underlying mechanisms are not clear, hepatic steatosis related to metabolic syndrome, a risk factor of erosive esophagitis, may provide a potential explanation. The TC/HDL-C ratio has been used to predict the risk of future coronary heart diseases. This practice is supported by several studies that have demonstrated that the TC/HDL-C ratio is the most significant predictor of future coronary heart disease, along with smoking and diabetes mellitus<sup>[37-39]</sup>. Our study showed that subjects with erosive esophagitis might have a higher cardiovascular risk than those without erosive esophagitis. The present study has several limitations. First, only the association between abnormal liver function and erosive esophagitis was analyzed, and no further evaluation was conducted to determine the relationship between erosive esophagitis and hepatic steatosis or chronic hepatitis B or C. Second, this study had a cross-sectional design, and therefore, only the associations between erosive esophagitis and metabolic syndrome, abnormal liver function, and

dyslipidemia could be determined. Further studies with a longitudinal design are required to evaluate their possible causal relationships.

In conclusion, metabolic syndrome and its components, such as central obesity, BP, and hypertriglyceridemia, are independent risk factors for erosive esophagitis. Abnormal liver function, including elevated AST, and cardiovascular risk factors were also found to be associated with erosive esophagitis. Further studies are needed to investigate the underlying mechanisms responsible for the relationship between abnormal liver function, cardiovascular risk, and erosive esophagitis.

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## COMMENTS

### Background

Erosive esophagitis is becoming more prevalent in Asia, but the underlying mechanism remains unknown. Obesity has been associated with erosive esophagitis. However, the associations of metabolic syndrome, its components, and liver function with erosive esophagitis are controversial.

### Research frontiers

Several previous studies have identified the risk factors of erosive esophagitis, including male sex, hiatal hernia, smoking, alcohol consumption, and obesity. This study further determined the associations between erosive esophagitis and metabolic syndrome, its components, and liver function.

### Innovations and breakthroughs

The present study demonstrated that metabolic syndrome, impaired liver function, and dyslipidemia were associated with erosive esophagitis.

### Applications

Individuals with metabolic syndrome and high cardiovascular risk, as defined by

a higher ratio of total cholesterol to high-density lipoprotein cholesterol (HDL-C), are at higher risk for erosive esophagitis. This finding may suggest that treating metabolic disorders can prevent or reduce the risk of erosive esophagitis. However, further studies are needed to confirm this finding.

### Terminology

Metabolic syndrome is a complex disorder comprising central obesity, high blood pressure, hyperglycemia, hypertriglyceridemia, and a low concentration of HDL-C. Cardiovascular risk, which is determined by a total cholesterol/HDL-C ratio > 5, correlates with the risk of cardiovascular events.

### Peer review

Authors undertook to characterize the correlation between erosive esophagitis and metabolic syndrome and its components, abnormal liver function, and abnormal lipoprotein profiles. This case-controlled study is well organized and has enough potential for publication.

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## Effect of early enteral combined with parenteral nutrition in patients undergoing pancreaticoduodenectomy

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### Abstract

**AIM:** To investigate the effect of early enteral nutrition (EEN) combined with parenteral nutritional support in patients undergoing pancreaticoduodenectomy (PD).

**METHODS:** From January 2006, all patients were given EEN combined with parenteral nutrition (PN) (EEN/PN group,  $n = 107$ ), while patients prior to this date were given total parenteral nutrition (TPN) (TPN group,  $n = 67$ ). Venous blood samples were obtained for a nutrition-associated assessment and liver function tests on the day before surgery and 6 d after surgery. The assessment of clinical outcome was based on postoperative complications. Follow-up for infectious and noninfectious complications was carried out for 30 d after hospital discharge. Readmission within 30 d after

discharge was also recorded.

**RESULTS:** Compared with the TPN group, a significant decrease in prealbumin (PAB) ( $P = 0.023$ ) was seen in the EEN/PN group. Total bilirubin (TB), direct bilirubin (DB) and lactate dehydrogenase (LDH) were significantly decreased on day 6 in the EEN/PN group ( $P = 0.006$ ,  $0.004$  and  $0.032$ , respectively). The rate of grade I complications, grade II complications and the length of postoperative hospital stay in the EEN/PN group were significantly decreased ( $P = 0.036$ ,  $0.028$  and  $0.021$ , respectively), and no hospital mortality was observed in our study. Compared with the TPN group (58.2%), the rate of infectious complications in the EEN/PN group (39.3%) was significantly decreased ( $P = 0.042$ ). Eleven cases of delayed gastric emptying were noted in the TPN group, and 6 cases in the EEN/PN group. The rate of delayed gastric emptying and hyperglycemia was significantly reduced in the EEN/PN group ( $P = 0.031$  and  $P = 0.040$ , respectively).

**CONCLUSION:** Early enteral combined with PN can greatly improve liver function, reduce infectious complications and delayed gastric emptying, and shorten postoperative hospital stay in patients undergoing PD.

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**Key words:** Enteral nutrition; Parenteral nutrition; Pancreaticoduodenectomy; Complications; Metabolism

**Core tip:** On the basis of our experience and the findings of previous studies, we investigated the effect of early enteral nutrition combined with parenteral nutritional support in patients undergoing pancreaticoduodenectomy enrolled in a retrospective controlled clinical trial. The results of this study showed that early enteral nutritional support combined with parenteral nutrition can greatly improve nutritional status and liver function, decrease the incidence of infectious complications and delayed gastric emptying, and shorten the length

of postoperative hospital stay.

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## INTRODUCTION

Pancreaticoduodenectomy (PD) is currently considered the treatment of choice for carcinoma of the periampullary region. Patients who are candidates for PD often have associated comorbidities such as diabetes, jaundice, and protein-energy malnutrition. PD results in loss of the gastric pacemaker and a partial pancreatic resection, and the physiologic consequence of this is a high incidence of postoperative malnutrition. PD is associated with a high incidence of postoperative complications, and this high rate of complications is likely to be multifactorial and may include overall nutritional debilitation<sup>[1]</sup>. Postoperative nutritional support therapy could ameliorate the clinical outcome in many types of surgical treatment, diminish the incidence of postoperative complications, and may be important in patients undergoing PD.

Recent research has shown that early postoperative enteral nutrition (EN) enhanced immunocompetence, decreased clinical infection rates, maintained gut structure and function, and can potentially attenuate catabolic stress responses in patients after surgery<sup>[2,3]</sup>. In addition, EN is believed to be safer and less expensive than parenteral nutrition (PN). However, postoperative total enteral feeding is associated with complications such as diarrhea, abdominal distention, and abdominal cramps. These symptoms worsen with increasing caloric intake and can lead to discontinuance of enteral feeding<sup>[2,4]</sup>. Gastroparesis is a frequent postoperative event following PD resection, and this often necessitates prolonged gastric decompression and enteral nutritional support<sup>[5]</sup>. Clinical data on postoperative early enteral nutrition (EEN) after PD are very limited. Therefore, on the basis of our experience and the findings of previous studies, we investigated the effect of EEN combined with parenteral nutritional support in patients undergoing PD enrolled in a retrospective controlled clinical trial.

## MATERIALS AND METHODS

### Patient selection

From January 2004 to June 2011, 196 patients underwent PD due to peri-ampullary tumors in the Department of Hepatobiliary Surgery at the Affiliated Drum Tower Hospital of Medical School of Nanjing University, China, where the authors work. Nineteen patients with manifest metabolic diseases (*e.g.*, diabetes mellitus and hyperthy-

roidism), severe hemorrhagic disease, ongoing infection, inflammatory bowel diseases or severe renal abnormality were excluded. Three patients with a history of gastric or pancreatic resection were also excluded, given the possible influence this procedure may have on the incidence of delayed gastric emptying. From January 2006, all patients were given EEN combined with PN (EEN/PN group,  $n = 107$ ), while patients prior to this date were given TPN (TPN group,  $n = 67$ ).

The primary endpoint of this study was the occurrence of major complications, and the secondary endpoint was 30 d after hospital discharge. The Nutrition Risk Screening 2002 (NRS 2002) scoring system<sup>[6]</sup> was used in this study, and the post-operative NRS 2002 score in all patients was  $\geq 3$ , indicating that all patients required nutritional support.

### Treatment

TPN was given 24 h/d for 5 d from the first day after PD. The nitrogen intake was 0.25 g/kg body weight per day, caloric intake was 125.4 kJ/kg per day and lipid intake was 1.1 g/kg per day. The nonprotein calories were given as dextrose (5.0 g/kg per day) and fat emulsion in a ratio of 2:1. The source of lipids was the standard lipid emulsion (20% emulsion, 5.5 mL/kg per day, long chain triglycerides: medium chain triglycerides 1:1, Huarui Pharmaceuticals, Jiangsu Province, China). Patients received 1.5 g amino acids/kg per day, administered as a commercially available compound amino acid solution (20% solution, Huarui Pharmaceuticals, Jiangsu Province, China). The proportion of nonprotein calories with nitrogen in both groups was 501.6 kJ/g. The PN solutions were prepared by a clinical pharmacist under aseptic conditions and adjusted to the weight of each patient. The amino acids, fat emulsion and dextrose mixture with electrolytes, vitamins and trace elements were administered *via* a central venous catheter. As soon as bowel function returned on 3-4 d after surgery, all patients were given liquid carbohydrate and cow's milk protein in equal amounts orally.

The surgical treatment was standardized, and lymph-node dissection was performed according to the definition provided by Pedrazzoli *et al*<sup>[7]</sup>. PD was performed by three groups of surgeons using the same technique. All patients received the same antibiotics postoperatively.

Patients in the EEN/PN group underwent preoperative placement of a conventional gastric tube. When gastrojejunostomy was complete, nasojejunal nutrition tubes were positioned (10 F, NUTRICIA Pharmaceutical Co., The Netherlands) from the nasal cavity to the output loops of the jejunum (approximately 20-25 cm with the help of a surgeon). The jejunum nutrition tube filar guide was then removed when the tube was in the correct position.

EN was given to patients in the EEN/PN group 24 h/d. An infusion of 100 mL of 5% glucose and sodium chloride injection (GNS) *via* a nasojejunal feeding tube was commenced within 24 h of surgery and 500 mL of 5% GNS was given on post-operative day 2 (POD2). On

**Table 1** Ranges and averages of calories, protein, fat and carbohydrates in the enteral and parenteral regimens in the early enteral nutrition/parenteral nutrition group

POD	Nutritional support	Calories (kJ)	Protein (g)	Fat (g)	Carbohydrates (g)
1	PN	7649.4 (6081.9-10282.8)	91.5 (72.8-123.0)	67.1 (53.3-90.2)	305.0 (242.5-410.0)
	EN	83.6	0	0	5
2	PN	7649.4 (6081.9-10282.8)	91.5 (72.8-123.0)	67.1 (53.3-90.2)	305.0 (242.5-410.0)
	EN	418	0	0	25
3	PN	6447.6 (4880.1-9081.0)	84.0 (65.3-115.5)	59.6 (45.8-82.7)	257.5 (195.0-362.5)
	EN	1201.8	7.5	7.5	47.5
4	PN	5559.4 (3991.9-8192.8)	76.5 (57.8-108.0)	52.1 (38.3-75.2)	235.0 (172.5-340.0)
	EN	2090	15	15	70
5	PN	3469.0 (1901.9-6102.8)	61.5 (42.8-93.0)	37.1 (23.3-60.2)	165.0 (102.5-270.0)
	EN	4180	30	30	140
6	PN	0	0	0	0
	EN	7649.4 (6081.9-10282.8)	54.9 (43.65-73.8)	54.9 (43.65-73.8)	256.2 (203.7-344.4)

Data are expressed as absolute average or average (range). POD: Post-operation day; PN: Parenteral nutrition; EN: Enteral nutrition.

POD3, 250 mL of Peptisorb liquid (2092 kJ/500 mL, NUTRICIA Pharmaceutical Co., the Netherlands) and 250 mL of 5% GNS were administered. Patients received 500 mL of Peptisorb liquid on POD4, and 1000 mL on POD5. From POD3, the PN recipe was adjusted according to the amount of EN, and the total caloric intake of PN and EN was 125.4 kJ/kg per day. PN was stopped on POD6, and patients in the EEN/PN group reached a maximum volume of total caloric intake following Peptisorb liquid (30 mL/kg per day). Oral intake started on POD7 and EN was stopped when the patients tolerated an intake of over 1000 kcal/d.

The body weight of the patients in this study varied from 48.1-84.6 kg, with an average body weight of 60.3 kg. Based on the range and average body weight, the ranges and averages of the calories, protein, fat and carbohydrates in the enteral and parenteral regimens in the EEN/PN group are listed in Table 1.

### Assessment

Venous heparin blood samples were obtained on 1 d (the day before surgery), and 6 d after surgery. Three types of measurement were carried out. First, a nutrition-associated assessment was carried out, which included serum albumin, prealbumin (PAB), total protein (TP), transferrin (TF) and total lymphocyte counts (TLCs). Serum albumin, PAB, total protein and TF were determined by an automatic biochemistry analyzer (HITACHI 7600, Hitachi Co., Tokyo, Japan). TLCs were determined using an automatic blood cell analyzer (COULTER STKS). The prognostic nutritional index (PNI) was calculated as follows:  $PNI = 0.005 \times TLC (10^6/L) + \text{albumin (g/L)}$ . The normal value of PNI is more than 50, and PNI values < 40 indicated malnutrition. Nitrogen balance was calculated as follows:  $N \text{ balance (g N/d)} = [\text{protein intake (g/d)/6.25}] - [\text{urinary urea (g/24 h)/2.14} + 3 \text{ g (nitrogen lost in skin and stool per day)}]$ . Second, a liver function assessment was carried out, which included serum total bilirubin (TB), direct bilirubin (DB), alanine aminotransferase (ALT), aspartate aminotrans-

ferase (AST) and lactate dehydrogenase (LDH) measurements. Liver function was determined by an automatic biochemistry analyzer (HITACHI 7600). Finally, clinical outcome was assessed based on postoperative complications. These complications were graded according to the Clavien-Dindo classification<sup>[8]</sup>, which was validated in pancreatic surgery<sup>[9]</sup>. Complications graded as III to V were considered as major. Pancreatic fistula and delayed gastric emptying were defined according to the International Study Group of Pancreatic Surgery (ISGPS)<sup>[10,11]</sup>. Operative mortality was defined as in-hospital death or death occurring within 30 d of discharge. Follow-up for infectious and noninfectious complications was carried out for 30 d after hospital discharge. Readmission within 30 d after discharge was also recorded.

### Statistical analysis

The results are expressed as mean  $\pm$  SD. Data were analyzed using the Statistical Analysis System (SAS). Differences between means were evaluated using the Student *t* test when normal distribution was confirmed by the Shapiro-Wilks test. When the hypothesis of normal distribution was rejected, differences between groups were tested by nonparametric statistics using the Mann-Whitney test for unpaired samples and Wilcoxon criteria for paired samples. Fisher's exact test was used for analysis of categorical values when appropriate. A *P* value of < 0.05 was considered significant.

## RESULTS

A total of 174 patients were enrolled in the study, 67 patients in the TPN group and 107 patients in the EEN/PN group. The mean age of the subjects was 53.2 years (range, 37-68 years). Demographic and preoperative clinical data, including age, sex, preoperative hemoglobin, preoperative albumin and the number of patients with jaundice or preoperative endoscopic nasal biliary drainage, are summarized in Table 2. No significant differences with respect to intraoperative factors, including opera-

**Table 2** Preoperative clinical data, intraoperative factors and histopathology of the patients enrolled in the study

	TPN group	EEN/PN group
Sex (male/female)	44/23	70/37
Age (yr)	52.8 ± 11.2	53.9 ± 10.6
Intraoperative factors		
Patients with jaundice (%)	79.1	83.2
Patients with preoperative ENBD (%)	50.7	48.6
Preoperative hemoglobin (g/L)	11.8 ± 1.0	12.4 ± 0.8
Preoperative albumin (g/L)	37.9 ± 3.1	36.8 ± 3.6
Duration of surgery (min)	345.1 ± 64.8	332.7 ± 56.6
Operative blood loss (mL)	648.4 ± 262.6	680.2 ± 193.7
Blood transfusion (%)	26.9	31.7
Histopathologic finding (n)		
Pancreatic head carcinoma	24	37
Distal cholangiocarcinoma	19	31
Periampullary adenocarcinoma	21	34
Duodenal adenocarcinoma	3	5

EEN: Early enteral nutrition; ENBD: Endoscopic nasal biliary drainage; PN: Prognostic nutritional; TPN: Total parenteral nutrition.

tion time, blood loss, number of patients who received blood transfusion and histopathological diagnosis, were observed between the two groups ( $P > 0.05$ ).

### Nutrition-associated assessment

No significant difference in the pre-operative nutrition-associated assessment was seen between the two groups. Compared with the results on the day before PD, a decrease in TP, PAB, TF and PNI was observed on day 6 after PD in all patients in this study, and a significant decrease in PAB in the TPN group ( $P < 0.05$ ) with no significant difference ( $P > 0.05$ ) in the EEN/PN group (Table 3).

Compared with the TPN group, a significant decrease in PAB ( $P = 0.02$ ) was seen in the EEN/PN group. However, no significant differences in TF, TP and PNI were noted between the two groups ( $P > 0.05$ ). Nitrogen balance was negative in both groups on day 6, with no significant difference between the two groups (Table 3).

### Liver function assessment

No significant differences in pre-operative liver function assessment were seen between the two groups. Compared with the results on the day before surgery, a significant decrease in ALT, AST, TB, DB and LDH was observed on 6 d in both groups ( $P < 0.05$ ), and a very significant decrease in TB and DB in the EEN/PN group ( $P < 0.01$ ).

Compared with the TPN group, a significant decrease in TB, DB and LDH was seen in the EEN/PN group ( $P < 0.05$ ). No significant differences in ALT and AST were observed between the two groups ( $P > 0.05$ ; Table 4).

### Clinical outcome

A prognostic score for major morbidity after PD has recently been proposed by Braga *et al.*<sup>[12]</sup>. The predictive risk score of major complications after PD in the two groups are listed in Table 5. There were no significant differences between the two groups in the score categorized in 4 risk

classes ( $P > 0.05$ ).

Table 6 shows the postoperative outcome in the two groups. Reoperation was necessary in 9 patients, and the causes of reoperation were early bleeding (1 case in the TPN group and 2 cases in the EEN/PN group), late bleeding (1 case in the EEN/PN group), abdominal abscess (2 cases in the TPN group and 2 cases in the EEN/PN group) and intestinal obstruction (1 case in the EEN/PN group). The causes of readmission in this study were intestinal obstruction (1 case in the TPN group and 2 cases in the EEN/PN group) and cholangitis (1 case in the TPN group and 1 case in the EEN/PN group). The rate of grade I complications, grade II complications and the length of postoperative hospital stay in the EEN/PN group were significantly reduced ( $P < 0.05$ ), and no hospital mortality was observed in this study (Table 6).

Postoperative complications are shown in detail in Table 7. There were 39 cases of infectious complications in the TPN group (8 cases of pneumonia, 7 cases of abdominal abscess, 5 cases of bile leak, 2 cases of pancreatic fistula, 4 cases of cholangitis, 8 cases of wound infection and 5 cases of urinary tract infection) and 42 cases in the EEN/PN group (6 cases of pneumonia, 6 cases of abdominal abscess, 7 cases of bile leak, 4 cases of pancreatic fistula, 3 cases of cholangitis, 10 cases of wound infection and 6 cases of urinary tract infection). Compared with the TPN group (58.2%), the rate of infectious complications in the EEN/PN group (39.3%) was significantly decreased ( $P < 0.05$ ). Eleven cases of delayed gastric emptying were observed in the TPN group, and 6 cases in the EEN/PN group. The rate of delayed gastric emptying and hyperglycemia was significantly decreased in the EEN/PN group ( $P < 0.05$ ). There were 29 cases of enteral-feeding-related complications in the EEN/PN group, including diarrhea, abdominal distention, and abdominal cramps. These symptoms were alleviated by slowing down the speed of enteral transfusion or by the administration of medications. None of the patients discontinued enteral feeding, and no enteral-feeding-related complications were noted in the TPN group.

## DISCUSSION

PD is associated with a high incidence of postoperative complications, and an overall morbidity rate of 48% can be anticipated at major centers<sup>[13]</sup>. The high rate of complications is likely to be multifactorial and may include overall nutritional debilitation, as most patients with periampullary tumors present with significant weight loss due to anorexia and malabsorption, and are expected to have a period of inadequate oral intake up to 10 d after surgery<sup>[14]</sup>. Compared with the results on the day before PD, a decrease in TP, PAB, TF, PNI and negative nitrogen balance were observed on day 6 in all patients in this study. Perioperative nutritional support can be beneficial in these patients in that it may reduce mortality and morbidity, and the length of hospital stay<sup>[15]</sup>.

Numerous studies have suggested that EN has sev-

**Table 3 Comparison of nutrition-associated assessment in the two groups (mean ± SD)**

	Normal value	Group	Day 1	Day 6	Decrease (days 1-6)
TP (g/L)	62-85	TPN	63.46 ± 7.24	59.92 ± 7.65	3.54 ± 1.72
		EEN/PN	64.11 ± 6.84	61.12 ± 6.83	2.99 ± 1.07
PAB (mg/L)	0-800	TPN	196.25 ± 64.32	116.52 ± 72.16 <sup>a</sup>	79.73 ± 35.32
		EEN/PN	190.15 ± 62.18	158.32 ± 62.46	31.83 ± 13.15 <sup>c</sup>
TF (g/L)	2.2-12	TPN	2.53 ± 0.76	2.20 ± 0.72	0.33 ± 0.61
		EEN/PN	2.46 ± 0.68	2.08 ± 0.81	0.38 ± 0.72
PNI	> 50	TPN	50.36 ± 9.14	43.12 ± 8.13	7.24 ± 7.40
		EEN/PN	51.62 ± 8.16	45.15 ± 9.52	6.47 ± 5.93
N-balance (g/d)		TPN	/	-(14.76 ± 6.03)	/
		EEN/PN	/	-(15.91 ± 7.85)	/

<sup>a</sup>*P* < 0.05 vs day 1; <sup>c</sup>*P* < 0.05 vs total parenteral nutrition (TPN) group. PAB: Prealbumin; PNI: Prognostic nutritional index; TP: Total protein; TF: Transferrin; EEN: Early enteral nutrition; PN: Parenteral nutrition.

**Table 4 Comparison of liver function in the two groups (mean ± SD)**

	Normal value	Group	Day 1	Day 6	Decrease (days 6-1)
ALT (μ/L)	5-40	TPN	138.2 ± 48.4	82.5 ± 42.3 <sup>a</sup>	55.7 ± 31.5
		EEN/PN	145.1 ± 39.2	77.4 ± 37.6 <sup>a</sup>	67.7 ± 36.2
AST (μ/L)	8-40	TPN	97.6 ± 36.2	55.1 ± 31.5 <sup>a</sup>	42.5 ± 26.2
		EEN/PN	102.3 ± 41.3	63.2 ± 36.3 <sup>a</sup>	39.1 ± 22.0
TB (μmol/L)	5-20.5	TPN	112.5 ± 37.5	66.2 ± 29.4 <sup>a</sup>	46.3 ± 34.3
		EEN/PN	106.8 ± 36.2	41.5 ± 34.1 <sup>b</sup>	65.3 ± 36.2 <sup>c</sup>
DB (μmol/L)	1.7-6.8	TPN	78.6 ± 30.2	38.1 ± 26.2 <sup>a</sup>	40.5 ± 21.3
		EEN/PN	81.7 ± 35.6	22.4 ± 16.2 <sup>b</sup>	59.3 ± 28.1 <sup>c</sup>
LDH (μ/L)	109-245	TPN	332.6 ± 89.4	264.3 ± 101.3 <sup>a</sup>	68.3 ± 51.2
		EEN/PN	316.2 ± 98.1	211.5 ± 86.2 <sup>a</sup>	104.7 ± 76.8 <sup>c</sup>

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs day 1; <sup>c</sup>*P* < 0.05 vs total parenteral nutrition (TPN) group. PN: Prognostic nutritional; EEN: Early enteral nutrition; TB: Total bilirubin.

**Table 5 Predictive risk score of major complications after pancreaticoduodenectomy in the two groups**

Predictor	Categories	Risk Score	TPN group	EEN/PN group
Pancreatic texture (%)	Hard	0	43	73
	Soft	4	24	34
Pancreatic duct diameter (%)	> 3 mm	0	48	81
	≤ 3 mm	1	19	26
Operative blood loss (%)	< 700 mL	0	55	81
	≥ 700 mL	4	12	26
ASA score (%)	I	0	31	55
	II	2	33	47
	III	6	3	5
Score categorized in 4 risk classes <i>n</i> (%)	0-3		23 (34.3)	38 (35.5)
	4-7		22 (32.8)	33 (30.8)
	8-11		19 (28.4)	31 (29.0)
	12-15		3 (4.5)	5 (4.7)

ASA: American Society of Anesthesiologist; EEN: Early enteral nutrition; TPN: Total parenteral nutrition; PN: Prognostic nutritional.

eral advantages over TPN. Early enteral feeding was shown to reduce postoperative septic complications in a meta-analysis of 8 prospective randomized trials, and improve glucose tolerance, protein kinetics and wound healing. Furthermore, EN is safer and less expensive than PN<sup>[16,17]</sup>. However, postoperative total enteral feeding is associated with complications such as diarrhea, abdomi-

**Table 6 Postoperative outcome in the two groups *n* (%)**

Group	TPN group	EEN/PN group
Complication Grade		
No complications	24 (35.8)	42 (39.2)
I	33 (49.3)	38 (35.5) <sup>a</sup>
II	38 (56.7)	42 (39.3) <sup>a</sup>
IIIa	7 (10.4)	10 (9.3)
IIIb	4 (6.0)	5 (4.6)
IVa	0 (0.0)	0 (0.0)
IVb	0 (0.0)	0 (0.0)
V (mortality)	0 (0.0)	0 (0.0)
Reoperation	3 (4.5)	6 (5.6)
Readmission	2 (3.0)	3 (2.8)
Postoperative hospital stay (d)	16.8 ± 6.2	13.2 ± 4.7 <sup>a</sup>

Numbers of single types of complications do not add up to the number of patients within the 2 groups, due to the possible occurrence of more types of complications in some patients. <sup>a</sup>*P* < 0.05 vs total parenteral nutrition (TPN) group. EEN: Early enteral nutrition; PN: Prognostic nutritional.

nal distention, and abdominal cramps. These symptoms worsen with increasing caloric intake and can lead to discontinuance of enteral feeding<sup>[2,3]</sup>. On the basis of these findings, we considered EEN combined with PN to be a better mode of postoperative nutritional support than total enteral feeding. On the first three days after surgery in this study, the amount of EN increased slowly to avoid severe gastrointestinal complications. Twenty-

**Table 7 Postoperative complications in the two groups *n* (%)**

Complications	TPN group	EEN/PN group
Pancreatic fistula	2 (3.0)	4 (3.7)
Grade A	0 (0.0)	0 (0.0)
Grade B	2 (3.0)	4 (3.7)
Grade C	0 (0.0)	0 (0.0)
Wound infection	8 (11.9)	10 (9.3)
Abdominal abscess	7 (10.4)	6 (5.6)
Bile leak	5 (7.5)	7 (6.5)
Cholangitis	4 (6.0)	3 (2.8)
Urinary tract infection	5 (7.5)	6 (5.6)
Pneumonia	8 (11.9)	6 (5.6)
Catheter-related sepsis	0 (0.0)	0 (0.0)
Gastrointestinal bleeding	4 (6.0)	5 (4.7)
Intraperitoneal bleeding	3 (4.5)	6 (5.6)
Delayed gastric emptying	11 (16.4)	6 (5.6)
Enteral-feeding-related complications	0 (0.0)	29 (27.1)
Abdominal cramps	0 (0.0)	6 (5.6)
Abdominal distention	0 (0.0)	11 (10.3)
Diarrhea	0 (0.0)	9 (8.4)
Vomiting	0 (0.0)	3 (2.8)
Hyperglycemia	12 (17.9)	6 (5.6)

Numbers of single types of complications do not add up to the number of patients within the 2 groups, due to the possible occurrence of more types of complications in some patients. EEN: Early enteral nutrition; PN: prognostic nutritional; TPN: Total parenteral nutrition.

nine cases in the EEN/PN group had enteral-feeding-related complications, these symptoms were alleviated by slowing down the speed of enteral transfusion or by the administration of medications, and none of the patients discontinued enteral feeding or dropped out of the study. PAB, which is more sensitive than albumin for evaluating protein synthesis in the liver due to its shorter half-life, was decreased on day 6 in all patients in this study. Compared with the TPN group, a significant decrease in PAB ( $P < 0.05$ ) was observed in the EEN/PN group.

Changes in transaminase and bilirubin are the most important indices for evaluating liver function in patients after PD. All patients in this study underwent PD to remove biliary obstruction, therefore, ALT, AST, TB, DB and LDH were significantly reduced. Lack of enteral feeding has several metabolic and endocrine consequences on intestinal and liver function. Experimental studies have shown that the fasted state reduces the secretion of several gastrointestinal hormones, such as cholecystokinin, gastrin and peptide YY. These hormones are instrumental in stimulating bile flow and gallbladder contraction, and for maintaining intestinal motility<sup>[18-20]</sup>. EN can also stimulate hepatic circulation and ameliorate liver function<sup>[21]</sup>. In the present study, a significant decrease in TB and DB in the EEN/PN group was observed compared with that in the TPN group.

EN preserved the gut flora architecture, prevented gastrointestinal mucosa atrophy, and inhibited microbial translocation from the gut to the blood stream<sup>[22,23]</sup>. Compared with the TPN group, the rate of infectious complication in the EEN/PN group was significantly decreased. The reduced length of postoperative hospital stay in the

EEN/PN group indicated that the time to complete recovery could be shortened by EEN support combined with PN. This may be explained by the lower number of complications.

Delayed gastric emptying (DGE) is also known as “gastroparesis”. DGE is not a fatal complication, but sometimes results in a significant prolongation of hospital stay and increased hospital costs. DGE has been reported to be affected by several factors including gastric dysrhythmias due to intra-abdominal complications, gastric atony after duodenal resection in response to a reduction in motilin levels, pylorospasm secondary to vagotomy, and angulation of the reconstructed alimentary tract<sup>[24-26]</sup>. Eleven cases of DGE were observed in the TPN group, and 6 cases in the EEN/PN group. EEN-support therapy significantly decreased the rate of DGE. One potential mechanism for the decreased rate of DGE due to EN may be the mechanical effects caused by the nasojejun tube or simply its presence across the anastomosis, which stimulates the motility of the stomach and jejunum, while another mechanism may be the stimulation of bowel movements by the input of nutritional liquids<sup>[27,28]</sup>.

In conclusion, we have shown that early enteral nutritional support combined with PN can greatly improve nutritional status and liver function, decrease the incidence of infectious complications and delayed gastric emptying, and shorten postoperative hospital stay in patients undergoing PD. Future randomized controlled trials are necessary to identify the correct application of PN and EN in patients receiving PD.

## COMMENTS

### Background

Pancreaticoduodenectomy (PD) is associated with a high incidence of postoperative complications. This high rate of complications is likely to be multifactorial and may include overall nutritional debilitation. Postoperative nutritional support therapy could ameliorate the clinical outcome in many types of surgical treatment and diminish the incidence of postoperative complications. The clinical data on postoperative early enteral nutrition (EEN) combined with parenteral nutrition (PN) after PD is very limited.

### Research frontiers

Recent research has shown that early postoperative enteral nutrition enhanced immunocompetence, lowered clinical infection rates, and maintained gut structure and function, and can potentially attenuate catabolic stress responses in patients after surgery. However, postoperative total enteral feeding is associated with complications such as diarrhea, abdominal distention, and abdominal cramps. These symptoms worsened with increasing caloric intake and can lead to discontinuance of enteral feeding.

### Innovations and breakthroughs

The authors investigated the effect of EEN combined with parenteral nutritional support in patients undergoing PD enrolled in a retrospective controlled clinical trial on the basis of their experience and the findings of previous studies. The results of this study show that early enteral nutritional support combined with PN can greatly improve nutritional status and liver function, decrease the incidence of infectious complications and delayed gastric emptying, and shorten postoperative hospital stay.

### Applications

The results of this study show that early enteral nutritional support combined with PN can greatly improve nutritional status and liver function, decrease

the incidence of infectious complications and delayed gastric emptying, and shorten postoperative hospital stay in patients undergoing PD. These findings are clinically relevant for guiding surgeons in the perioperative administration of medications during PD.

### Peer review

This is an interesting study which is well written and referenced. It is a non-randomized retrospective study of the effect of EEN combined with parenteral nutritional support for patients receiving PD. PD is a major surgical procedure for the treatment of periampullary tumors which will result in a high incidence of complications and postoperative malnutrition, but nutritional support can improve patient's malnutrition and diminish the incidence of postoperative complications.

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## Association between *UCP3* gene polymorphisms and nonalcoholic fatty liver disease in Chinese children

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and 103 females) and 200 healthy individuals who served as controls (control, 109 males and 91 females), aged between 6 and 16 years were enrolled in this study. The four non-synonymous single nucleotide polymorphisms (SNPs) in the *UCP3* gene polymorphisms of rs1726745, rs3781907, rs11235972 and rs1800849, were genotyped using MassArray. Body mass index (BMI), waist and hip circumference, blood pressure (BP), fasting blood glucose (FBG), insulin and lipid profiles were measured and B-ultrasound examination was performed in all subjects.

**RESULTS:** NAFLD patients showed risk factors for metabolic syndrome: elevated BMI, waist-to-hip ratio, BP, FBG, homeostasis model assessment-estimated insulin resistance, total triglyceride, total cholesterol and low-density lipoprotein-cholesterol, while decreased high-density lipoprotein-cholesterol level compared with the control group. The GG genotype distributions of rs11235972 in the NAFLD group differed significantly from that in the control group. We found that waist circumference between CC ( $58.76 \pm 6.45$  cm) and CT+TT ( $57.00 \pm 5.59$  cm), and hip circumference between CC ( $71.28 \pm 7.84$  cm) and CT+TT genotypes ( $69.06 \pm 7.75$  cm) were significantly different with and without rs1800849 variation ( $P < 0.05$ ).

**CONCLUSION:** A higher prevalence of rs11235972 GG genotype was observed in the NAFLD group compared with the control group. No differences were observed for the other SNPs. However, there was a significant difference in body height in addition to waist and hip circumference between the CC (mutant type group) and CT+TT group with and without rs1800849 variation.

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**Key words:** Nonalcoholic fatty liver disease; Uncoupling protein 3; Single nucleotide polymorphisms

**Core tip:** There are few population-based prevalence

### Abstract

**AIM:** To confirm the hypothesis that polymorphisms of the uncoupling protein 3 (*UCP3*) gene are associated with the occurrence of nonalcoholic fatty liver disease (NAFLD).

**METHODS:** A total of 250 NAFLD patients (147 males

studies of pediatric nonalcoholic fatty liver disease (NAFLD). Uncoupling protein 3 (*UCP3*) is considered to be associated with obesity, given the role for *UCP3* in the regulation of energy and lipid metabolism. This is the first study to report that there significant difference of body height, waist and hip circumference between CC (mutant type group) and CT+TT group with and without rs1800849 variation were found. This study confirmed the hypothesis that polymorphisms of the *UCP3* are associated with the occurrence of NAFLD. These variations could be useful for the diagnosis and/or prognosis of NAFLD.

Xu YP, Liang L, Wang CL, Fu JF, Liu PN, Lv LQ, Zhu YM. Association between *UCP3* gene polymorphisms and nonalcoholic fatty liver disease in Chinese children. *World J Gastroenterol* 2013; 19(35): 5897-5903 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i35/5897.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5897>

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathologic condition characterized by abnormal lipid deposition in hepatocytes in the absence of excess alcohol intake. NAFLD comprises a wide spectrum of liver damage, including simple steatosis, steatohepatitis, fibrosis or even cirrhosis of the liver<sup>[1]</sup>. NAFLD does not only impact adults, but is also one of the major causes of liver diseases in children<sup>[2]</sup>. There are few population-based prevalence studies of pediatric NAFLD. Some studies have suggested a prevalence of 2.6%-9.6% for suspected NAFLD among children and adolescents in the United States<sup>[3,4]</sup> and Asia<sup>[5,6]</sup>. NAFLD has been shown to be associated with metabolic syndrome (MetS), which comprises obesity, type 2 diabetes, dyslipidemia and high blood pressure (BP) with insulin resistance being the central mechanism<sup>[7,8]</sup>. Theoretically, many variations in candidate genes related to MetS may contribute to the pathogenesis of NAFLD, such as genes related to insulin resistance and genes influencing hepatic free fatty acid metabolism. Elucidation of genetic factors that predispose an individual to NAFLD may lead to the development of non-invasive biomarkers for the early diagnosis of NAFLD and may allow early preventive and therapeutic strategies for those at the high risk.

Uncoupling protein 3 (*UCP3*) gene is located on chromosome 11q13. *UCP3* is a mitochondrial anion carrier protein with a highly selective expression in skeletal muscle, a major site of thermogenesis in humans, which makes an attractive target for studies into the regulation of body weight. Reduced expression of *UCP3* decreases energy expenditure and increased expression of *UCP3* mRNA in muscle is related to an increase in the metabolic rate and to a lower body mass index (BMI)<sup>[9,10]</sup>. Therefore, *UCP3* may be involved in obesity, given the role of *UCP3* in the regulation of energy and lipid metabolism.

Genetic variants of *UCP3* have been identified, and specifically polymorphisms of 55C/T may impact type 2 diabetes mellitus (T2DM), obesity and weight gain<sup>[11-13]</sup>. This study confirmed the hypothesis that polymorphisms of the *UCP3* are associated with the occurrence of NAFLD. These variations could be useful for the diagnosis and/or prognosis of NAFLD, although the functional significance of *UCP3* polymorphisms is not clear.

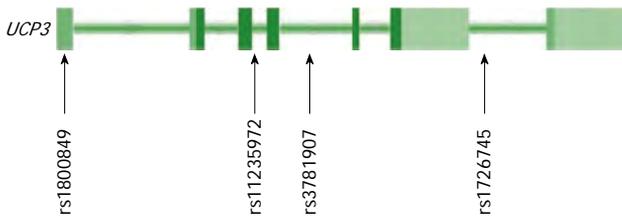
## MATERIALS AND METHODS

### Subjects

A total of 250 NAFLD children and 200 healthy individuals (controls), aged between 6 and 16 years were enrolled in this study. NAFLD children (147 males and 103 females) were referred to our endocrinology department from January 2006 to September 2011; NAFLD was defined according to the revised definition and treatment guidelines for NAFLD by the Chinese Hepatology Association in February 2006<sup>[14,15]</sup>, and was diagnosed by means of a protocol using clinical, laboratory and ultrasound examinations in combination. In this study, NAFLD was diagnosed as a diffusely echogenic change on liver B-ultrasonography (fatty infiltration in liver), with or without elevated serum aminotransferase levels, and other factors which can cause liver fatty infiltration or aminotransferase elevation, such as infectious hepatitis (hepatitis B and C, Epstein-Barr virus infection), drug-induced hepatitis, and some metabolic diseases were excluded. None of the subjects had a history of alcohol consumption. Blood samples ( $n = 200$ ) were also obtained from healthy individuals, who served as controls (109 males and 91 females) in 2011 from the Department of Child Health Care, The Affiliated Yuying Children's Hospital of Wenzhou Medical University and Ningbo Women and Children's Hospital. The protocol was approved by the Medical Ethics Committee of The Children's Hospital of Zhejiang University School of Medicine. Written informed consent were obtained from parents (or guardians) and children (where appropriate).

### Laboratory assessment

The weight and height of the subjects were measured with a calibrated scale after removing shoes and heavy clothing, if any. BMI is calculated by taking the ratio of weight in kilogram and the square of height in meter. Waist was measured at the midpoint between the lower border of the rib cage and the iliac crest. Hip circumference was determined at the widest circle of the bottom. Venous blood samples were obtained from the subjects after an overnight fasting (12 h) for the measurement of fasting blood glucose (FBG), fasting insulin (FIN), total triglyceride (TG), total cholesterol (TCHO), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C), alanine transaminase (ALT) and aspartate aminotransferase (AST). All laboratory biochemical parameters were measured in a conventional automated analyzer.



**Figure 1** Locus of the human *UCP3* gene in 11q13. The uncoupling protein 3 (*UCP3*) gene consists of seven exons separated by six introns. Boxes indicate exons, while lines indicate introns and intergenic regions. Mark the polymorphism locations.

**Liver ultrasound examination**

Sagittal hepatic sections that encompassed longitudinal images of the right liver lobe and the ipsilateral kidney were obtained. Liver-kidney contrast with two other well-known ultrasonographic findings of fatty liver, vascular blurring and deep attenuate, enabled us to grade fatty change semi-quantitatively<sup>[15]</sup>. Ultrasound examination was carried out and blinded to laboratory values on the same equipment (GE, LOGIC 500), using a convex 3.5-5.0 MHz probe. NAFLD and healthy individuals underwent liver ultrasound examination.

**DNA preparation and single nucleotide polymorphism genotyping**

Using information on single nucleotide polymorphism (SNP) allelic frequencies from the website of the National Center for Biotechnology Information (NCBI) and the SNP browser software 3.0 (Applied Biosystems, Branchburg, NJ, United States), SNPs on the human *UCP3* gene with minor allele frequencies > 30% were selected. SNPs with relatively high minor allele frequencies have been shown to be very useful as genetic markers for genetic association studies. We selected four non-synonymous SNPs in the *UCP3* gene: polymorphisms of rs1726745, rs3781907, rs11235972, and rs1800849 (Figure 1). Genomic DNA was extracted from blood samples collected from each subject. Polymorphisms were genotyped using an automated platform MassARRAY (Sequenom, San Diego, CA). Polymerase chain reaction for the DNA sequence containing the target SNP was performed. The products were extended one base in SNP sites using the SNP specific primer. The products were applied into the MassARRAY SpectroCHIP array and crystallized with matrix in the chip. The crystal containing chip was moved to the mass spectrometer vacuum tube and excited using an instantaneous nanosecond (10<sup>-9</sup> s) laser. The molecular of matrix absorb the radiation energy, which lead to energy accumulation causing crystal matrix sublimation, DNA molecule desorption and transformation to metastable ions.

**Statistical analysis**

Quantitative data with normal distribution were presented as mean ± SD. Categorical variables were expressed as a percentage and examined using the  $\chi^2$  test and Fisher's tests. Hardy-weinberg test was performed to

**Table 1** Demographic and biochemical features of patients with nonalcoholic fatty liver disease and normal controls

	NAFLD (n = 250)	Controls (n = 200)	P value
Gender (M/F)	147/103	109/91	0.36
Age (yr)	10.78 ± 2.07	10.63 ± 2.22	0.47
Body height (cm)	148.28 ± 11.77	141.22 ± 12.91	0.00
Body weight (kg)	62.82 ± 15.17	34.72 ± 10.28	0.00
BMI (kg/m <sup>2</sup> )	28.13 ± 3.50	17.05 ± 2.16	0.00
SBP (mmHg)	114.02 ± 11.55	91.61 ± 9.68	0.00
DBP (mmHg)	68.57 ± 8.63	66.43 ± 7.67	0.01
Waist (cm)	89.84 ± 9.74	58.07 ± 7.04	0.00
Hip (cm)	93.85 ± 8.66	70.09 ± 7.85	0.00
WHR	0.95 ± 0.06	0.83 ± 0.05	0.00
FBG (mmol/L)	4.99 ± 0.41	4.91 ± 0.38	0.04
TCHO (mmol/L)	4.40 ± 0.89	4.14 ± 0.66	0.00
HDLc (mmol/L)	1.33 ± 0.51	1.52 ± 0.30	0.00
LDLc (mmol/L)	2.53 ± 0.72	2.19 ± 0.56	0.00
TG (mmol/L)	1.41 ± 0.84	0.80 ± 0.31	0.00
ALT (mmol/L)	72.70 ± 70.16	17.14 ± 10.48	0.00
AST (mmol/L)	47.26 ± 36.61	25.22 ± 6.53	0.00
FIN (mIU/L)	20.49 ± 17.25	6.98 ± 3.59	0.00
HOMA-IR	4.57 ± 3.92	1.52 ± 0.78	0.00

Data are expressed as absolute mean ± SD. Analysis was conducted using  $\chi^2$  test and *t* test. NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic pressure; WHR: Waist-to-hip ratio; FBG: Fasting blood glucose; TCHO: Total cholesterol; HDLc: High-density lipoprotein-cholesterol; LDLc: Low-density lipoprotein-cholesterol; TG: Total triglyceride; ALT: Alanine transaminase; AST: Aspartate aminotransferase; FIN: Fasting insulin; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; M: Male; F: Female.

calculate allelic frequencies using the  $\chi^2$  test. Multivariate logistic regression analysis by using stepwise selection was constructed to determine which of the potential risk factors of NAFLD were. Given the BMI, HOMA-IR, ALT, TCHO, rs1726745, rs3781907, rs11235972, and rs1800849 risk factors relative to the potential number of variables in our model, only those variables that had the highest possibility for independent prediction of outcome in our logistic regression were included. Multivariate logistic regression analysis was performed to estimate the OR and 95%CI for the potential risk factors of NAFLD. The statistical significance of means was estimated by independent *t* test. HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5. A *P* value of < 0.05 was regarded as statistical significant. All data analysis was done using the SPSS for windows (version 13.0; SPSS, Inc., Chicago, IL, United States). Haploview software (Cambridge, MA, United States) was used to screen tag SNPs.

**RESULTS**

The clinical features of the NAFLD and control groups are shown in Table 1. There was no significant difference in age and gender (*P* > 0.05). NAFLD patients showed most of the risk factors for the MetS: elevated BMI, waist-to-height ratio (WHR), BP, FBG, HOMA-IR, TG, TCHO and LDL, while decreased HDL level compared to control group.

**Table 2** The genotypic distributions of the four loci in the *UCP3* gene *n* (%)

	<i>n</i>	Genotypes			<i>P</i> value	Allele Frequency		<i>P</i> value
		Homozygous wild-type	Homozygous mutant-type	Heterozygous mutant-type		Mutant-type	Wild-type	
rs1726745		GG	AA	AG		A	G	
NAFLD	249	40 (16.1)	102 (41.0)	107 (43.0)	0.59	311 (62.4)	187 (37.6)	0.29
Controls	200	37 (18.5)	73 (36.5)	90 (45.0)		236 (59.0)	164 (41.0)	
rs3781907		TT	CC	CT		C	T	
NAFLD	239	87 (36.4)	42 (17.6)	110 (46.0)	0.23	194 (40.6)	284 (59.4)	0.19
Controls	198	57 (28.8)	37 (18.7)	104 (52.5)		178 (44.9)	218 (55.0)	
rs11235972		GG	AA	AG		A	G	
NAFLD	236	130 (55.1)	26 (11.0)	80 (33.9)	0.03	132 (28.0)	340 (72.0)	0.28
Controls	198	90 (45.5)	16 (8.0)	92 (46.5)		124 (31.3)	272 (68.7)	
rs1800849		CC	TT	TC		T	C	
NAFLD	249	133 (53.4)	25 (10.0)	91 (36.5)	0.14	141 (28.3)	357 (71.7)	0.54
Controls	199	92 (46.2)	16 (8.0)	91 (45.7)		123 (30.9)	275 (69.1)	

NAFLD: Nonalcoholic fatty liver disease.

**Table 3** Multivariate regression analysis for risk factors of nonalcoholic fatty liver disease (*n* = 449)

	<i>B</i>	<i>SE</i>	Wald	<i>df</i>	Sig	Exp ( <i>B</i> )	95%CI for EXP ( <i>B</i> )	
							Lower	Upper
BMI	1.98	0.77	6.67	1	0.01	7.24	1.61	32.53
HOMA-IR	1.10	0.47	5.54	1	0.02	3.01	1.20	7.55
ALT	0.05	0.06	0.54	1	0.46	1.05	0.93	1.19
TCHO	-0.64	0.88	0.53	1	0.47	0.53	0.10	2.94
rs1800849	-7.28	40192.97	0	1	1	0	0	-
rs11235972	5.15	40192.97	0	1	1	172.72	0	-
rs3781907	1.09	2.01	0.30	1	0.59	2.98	0.06	151.89
rs1726745	-0.67	4.42	0.02	1	0.88	0.51	0	2933.6

TCHO: Total cholesterol; ALT: Alanine transaminase; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; BMI: Body mass index.

The distributions of the four SNPs (rs1726745, rs3781907, rs11235972 and rs1800849) obeyed the Hardy-Weinberg equilibrium in all subjects. The genotypic distributions of the four loci in the *UCP3* gene are shown in Figure 1 and Table 2. The genotype distributions of rs11235972 in the NAFLD group differed significantly from that in the control group.

When all variables were put into multivariate logistic regression analysis, higher BMI and HOMA-IR were risk factors for the development of NAFLD (Table 3). The clinical features in subjects with or without rs1800849 variation are shown in Table 4. There were no significant differences in anthropometric and biomedical variables with or without rs11235972 variation in the NAFLD and in the control groups, respectively. We found body height between the CC (142.93 ± 13.08 cm) and CT+TT (139.38 ± 12.10 cm), waist circumference between the CC (58.76 ± 6.45 cm) and CT+TT (57.00 ± 5.59 cm) and hip circumference between the CC (71.28 ± 7.84 cm) and CT+TT (69.06 ± 7.75 cm) genotypes were significantly different in the control group with and without rs1800849 variation (*P* < 0.05).

When all genotypes of the four loci were entered into haplotype analysis using Haploview, only two haplotypes' alleles *i.e.*, GC and AT frequencies were accepted for assessment between the NAFLD and control groups. How-

ever, this did not reach the significance as an independent risk factor for NAFLD (Table 4 and Figure 2).

## DISCUSSION

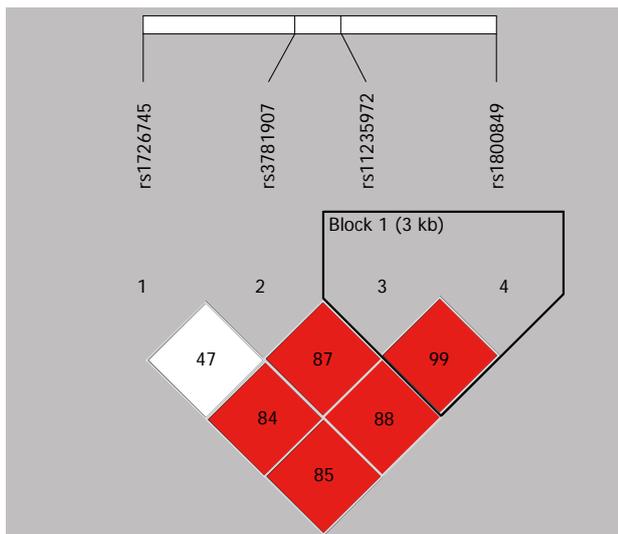
Previous reports have demonstrated that the prevalence of NAFLD increased 10%-80% in individuals with obesity, 35%-90% in individuals with T2DM, 30%-56% in individuals with hypertension, and 26%-58% in individuals with dyslipidemia<sup>[16-18]</sup>. The prevalence of the MetS among subjects with NAFLD is 17%-36%, depending on gender and criteria used<sup>[19]</sup>. In the present study, we found that an increased risk of NAFLD was significantly associated with BMI, WHR, BP, FBG, HOMA-IR, TG, TCHO and LDL, and decreased HDL level. In agreement with these studies, Iacobellis *et al.*<sup>[20]</sup> reported that a BMI evaluation may be useful in identifying those children at higher risk for disease progression. In the present study, children with NAFLD had an elevated WHR, a surrogate marker for visceral fat. Visceral fat is closely correlated with hepatic TG content, elevated ALT, liver inflammation, and fibrosis<sup>[21-23]</sup>. Central obesity is a better measure of predisposition to insulin resistance, and is more closely associated with NAFLD.

*UCP3* is considered to be associated with obesity, given the role for *UCP3* in the regulation of energy and

**Table 4** Demographic and biochemical features of subjects with and without rs1800849 variation (mean ± SD)

	Control			NAFLD		
	CC (n = 92)	CT+TT (n = 107)	P value	CC (n = 133)	CT+TT (n = 116)	P value
Gender (M/F)	48/44	60/47	0.582	103/30	90/26	0.979
Body height (cm)	142.93 ± 13.08	139.38 ± 12.10	0.048	148.82 ± 12.50	147.83 ± 10.82	0.510
Body weight (kg)	35.65 ± 9.69	33.29 ± 8.51	0.070	64.42 ± 16.86	61.14 ± 12.74	0.083
BMI (kg/m <sup>2</sup> )	17.11 ± 2.00	16.86 ± 1.80	0.358	28.45 ± 3.75	27.79 ± 3.17	0.141
SBP (mmHg)	91.59 ± 8.89	91.29 ± 9.80	0.824	113.32 ± 11.74	114.84 ± 11.36	0.301
DBP (mmHg)	66.71 ± 7.11	66.13 ± 8.16	0.595	68.27 ± 9.13	68.91 ± 8.08	0.561
Waist (cm)	58.76 ± 6.45	57.00 ± 5.59	0.040	90.78 ± 10.58	88.87 ± 8.57	0.123
Hip (cm)	71.28 ± 7.84	69.06 ± 7.75	0.047	94.31 ± 9.51	93.47 ± 7.64	0.483
WHR	0.83 ± 0.05	0.83 ± 0.04	0.833	0.95 ± 0.07	0.95 ± 0.05	0.559
FBG (mmol/L)	4.91 ± 0.35	4.92 ± 0.40	0.888	5.02 ± 0.44	4.96 ± 0.36	0.245
TG (mmol/L)	4.11 ± 0.65	4.17 ± 0.67	0.519	4.32 ± 0.82	4.48 ± 0.96	0.165
HDL (mmol/L)	1.48 ± 0.29	1.55 ± 0.30	0.128	1.33 ± 0.58	1.35 ± 0.40	0.743
LDL (mmol/L)	2.18 ± 0.52	2.19 ± 0.58	0.950	2.50 ± 0.70	2.56 ± 0.75	0.457
ALT (mmol/L)	16.85 ± 6.44	16.48 ± 8.92	0.740	54.37 ± 2.07	51.43 ± 2.28	0.574
AST (mmol/L)	23.90 ± 5.54	26.07 ± 6.56	0.013	47.53 ± 36.24	46.91 ± 37.35	0.896
FINS	7.20 ± 3.34	6.62 ± 3.35	0.226	17.13 ± 2.11	14.86 ± 2.12	0.140
HOMA-IR	1.58 ± 0.75	1.45 ± 0.74	0.231	3.80 ± 2.14	3.27 ± 2.12	0.117

Analysis was conducted using *t* test. Statistically significant differences between groups are shown in bold. NAFLD: Nonalcoholic fatty liver disease; M: Male; F: Female; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic pressure; WHR: Waist-to-hip ratio; FBG: Fasting blood glucose; HDL: High-density lipoprotein-cholesterol; LDL: Low-density lipoprotein-cholesterol; TG: Total triglyceride; ALT: Alanine transaminase; AST: Aspartate aminotransferase; FIN: Fasting insulin; HOMA-IR: Homeostasis model assessment-estimated insulin resistance.



**Figure 2** Linkage Disequilibrium mapping around the uncoupling protein 3 between nonalcoholic fatty liver disease and control groups.

lipid metabolism. However, the effect of genetic polymorphisms in *UCP3* on the pathogenesis of NAFLD has not been clearly documented. In our study, the GG genotype distribution of rs11235972 in the NAFLD group differed significantly from the control group. The results from the literature are controversial. Yoon *et al.*<sup>[24]</sup> genotyped 6 polymorphisms of *UCP3* among overweight female subjects (*n* = 458), and genetic effects on BMI and changes after a very low calorie diet were examined. They found that several polymorphisms in the *UCP2-3* gene cluster showed associations with changes in BMI and fat mass. Hamada *et al.*<sup>[25]</sup> determined whether the *UCP3*-55 C/T SNP was associated with obesity according to the criteria for Japanese (BMI ≥ 25 kg/m<sup>2</sup>) and

**Table 5** Haplotype frequencies of rs1800849, rs11235972, rs3781907, rs1726745

Block	Frequencies		χ <sup>2</sup>	P value
	NAFLD	Control		
GC	0.712	0.682	0.901	0.343
AT	0.282	0.307	0.702	0.402

NAFLD: Nonalcoholic fatty liver disease.

serum HDL-C levels in the general population. Subjects with the T/T genotype had significantly higher HDL-C levels than those with the C/C genotype or C/T genotype. Furthermore, subjects with the T/T genotype had a significantly lower BMI than those with the C/C genotype<sup>[25]</sup>. Salopuro *et al.*<sup>[26]</sup> found that the *UCP3* gene variant rs3781907 was associated with increased serum TCHO and LDL cholesterol levels. The rs1726745, rs11235972 and rs1800849 variants in the *UCP3* gene are associated with serum total and LDL-cholesterol at baseline. However, de Luis *et al.*<sup>[27]</sup> did not demonstrate an association between the -55CT polymorphism of the *UCP3* gene and fat distribution in obese patients. There might be true variability in the association among different populations, particularly different ethnic groups.

A common promoter polymorphism has also been identified in the *UCP3* gene (rs1800849), a rare allele associated with obesity in a recessive manner in several studies<sup>[10-12]</sup>. Moreover, the rs1800849 allele is associated with a higher WHR<sup>[28]</sup>, but no association between rs1800849 and WHR existed in the current study. We showed a significant difference in height, and waist and hip circumference between the CC (mutant type group) and CT+TT group with and without rs1800849 variation (Table 5).

One limitations in this study was that we used abdominal ultrasonography to diagnose NAFLD, although validation ultrasonography has a sensitivity of 91.7% and a specificity of 100%<sup>[29]</sup>. The diagnosis of NAFLD was based on ultrasound and was not confirmed by liver biopsy due to the invasive procedure usually not initially performed and ethical considerations. Thus, surrogate markers are commonly used, such as transaminases and imaging techniques. Computed tomography is more specific but is not used for screening of fatty liver in obese children. Magnetic tomography is more useful in adults and not appropriate for children due to ionizing radiation. Magnetic resonance imaging and 1H-MRS have the greatest accuracy to determine hepatic fat content, but are rarely used due to high costs. A higher prevalence of the rs11235972 GG genotype was noted in the NAFLD group compared with the control group; no differences were observed for the other SNPs. BMI and HOMA-IR increased the risk of NAFLD. Moreover, no increased risk for developing NAFLD was found to be associated with the rs1800849 variant based on multivariate analysis. A significant difference in height, and waist and hip circumference between the CC and CT+TT group with and without rs1800849 variation was demonstrated.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathologic condition characterized by abnormal lipid deposition in hepatocytes in the absence of excess alcohol intake. There are few population-based prevalence studies of pediatric NAFLD. Elucidation of genetic factors that predispose an individual to NAFLD may lead to development of non-invasive biomarkers for the early diagnosis of NAFLD.

### Research frontiers

Uncoupling protein 3 (*UCP3*) is considered to be associated with obesity, given the role for *UCP3* in the regulation of energy and lipid metabolism. However, the effect of genetic polymorphisms in *UCP3* on the pathogenesis of NAFLD has not been clearly documented.

### Innovations and breakthroughs

Theoretically, many variations in candidate genes related to MetS may contribute to the pathogenesis of NAFLD. This is the first study to report that there significant difference of body height, waist and hip circumference between CC (mutant type group) and CT+TT group with and without rs1800849 variation were found.

### Applications

This study confirmed the hypothesis that polymorphisms of the *UCP3* are associated with the occurrence of NAFLD. These variations could be useful for the diagnosis and/or prognosis of NAFLD.

### Terminology

*UCP3* gene is located on chromosome 11q13. *UCP3* is a mitochondrial anion carrier protein with a highly selective expression in skeletal muscle, a major site of thermogenesis in humans, which makes an attractive target for studies into

the regulation of body weight.

## Peer review

The authors examined the polymorphisms of the *UCP3* gene associated with the occurrence of NAFLD. Higher rs11235972 GG genotype prevalence has been observed in the NAFLD group compared with the control group. There significant difference of body height, waist and hip circumference between CC (mutant type group) and CT+TT group with and without rs1800849 variation were found. This is an interesting manuscript about NAFLD in children and *UCP3* polymorphisms. The manuscript has adequate methodology and is good written.

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## Influence of chronic HBV infection on superimposed acute hepatitis E

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### Abstract

**AIM:** To investigate the influence of chronic hepatitis B virus (HBV) infection [based on the status of hepatitis B e antigen (HBeAg), HBV DNA, and cirrhosis] on superimposed acute hepatitis E.

**METHODS:** A total of 294 patients were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University, from January 2003 to January 2012. The patients were classified into two groups: an HBV + hepatitis E virus (HEV) group (a group with chronic HBV infection that was superinfected with acute hepatitis E,  $n = 118$ ) and an HEV group (a group with acute hepatitis E,  $n = 176$ ). We retrospectively analyzed and compared the clinical features of the two groups. Statistical analyses were performed using the  $\chi^2$  test or Fisher's exact test for categorical variables and the Student's  $t$  test for

continuous variables. A  $P$  value  $< 0.05$  was considered statistically significant.

**RESULTS:** The peak values of prothrombin time, serum total bilirubin, and Model for End-Stage Liver Disease scores were significantly higher in the HBV + HEV group. More patients in the HBV + HEV group had complications (39.8% vs 16.5%,  $P = 0.000$ ) and developed liver failure (35.6% vs 8.5%,  $P = 0.000$ ). Additionally, the mortality of the HBV + HEV group was significantly higher (20.3% vs 7.4%,  $P = 0.002$ ). Further analysis of the HBV + HEV group showed that there were no significant differences in complication occurrence, liver failure incidence, or mortality between patients with different HBeAg and HBV DNA statuses. However, in patients with underlying cirrhosis, complication occurrence and liver failure incidence significantly increased. In total, 12.7% of the patients in the HBV + HEV group received anti-HBV treatment, but this therapy failed to reduce mortality in patients who developed liver failure.

**CONCLUSION:** The presence of underlying cirrhosis in chronic HBV infection results in more severe clinical outcomes with superimposed acute hepatitis E. Anti-HBV treatment cannot improve the prognosis of liver failure caused by HBV-HEV superinfection.

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**Key words:** Chronic hepatitis B virus infection; Acute hepatitis E; Superinfection; Clinical profile; Anti-hepatitis B virus treatment

**Core tip:** Previous studies have shown that chronic hepatitis B virus (HBV) infection has a negative impact on superimposed acute hepatitis E. However, it remains unknown whether the disease severity of acute hepatitis E correlates with the underlying HBV replication status or with liver histological lesions. Our study

showed that the disease severity of acute hepatitis E correlated not with the HBV replication status (based on the status of hepatitis B e antigen and HBV DNA), but rather with the presence of underlying cirrhosis. This finding raised the question of whether anti-HBV treatment improves the outcome of liver failure caused by HBV-hepatitis E virus superinfection. We found that anti-HBV treatment could not improve the prognosis of such liver failure.

Cheng SH, Mai L, Zhu FQ, Pan XF, Sun HX, Cao H, Shu X, Ke WM, Li G, Xu QH. Influence of chronic HBV infection on superimposed acute hepatitis E. *World J Gastroenterol* 2013; 19(35): 5904-5909 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5904.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5904>

## INTRODUCTION

Infection by hepatitis B virus (HBV) is a serious public health problem worldwide. Two billion people worldwide have been infected with HBV, including more than 240 million cases of chronic infection<sup>[1,2]</sup>. During the chronic course of HBV infection, there is a chance that patients may be sporadically superinfected with other viruses, such as hepatitis E virus (HEV). HEV is mainly endemic in tropical and subtropical developing countries, including China. Studies of serum epidemiology in China showed that HEV superinfection in patients with chronic hepatitis B is present in 17.6% of these patients<sup>[3]</sup>.

HEV generally causes an acute, self-limiting illness, followed by a complete recovery. Recent studies have shown that HEV can result in severe disease in patients with underlying chronic HBV infection and even liver failure<sup>[4-7]</sup>. In the chronic course of HBV infection, there are different statuses of hepatitis B e antigen (HBeAg) and HBV DNA, and certain patients have a higher probability of developing cirrhosis. No previous studies are available regarding whether these different chronic statuses have different influences on the superinfection of HBV and HEV.

Liver failure related to HBV activation remains a rapidly progressive and frequently fatal condition. Traditional treatment is generally supportive. International HBV treatment guidelines recommend initiating nucleos(t)ide analogs as early as possible in this patient population<sup>[8,9]</sup>. Studies on the efficacy of nucleoside analogs have been emerging in recent years. Recent studies have shown that anti-HBV treatment could improve the outcome of this patient population<sup>[10-13]</sup>. Superinfection with HEV is another common cause of liver failure in patients with chronic HBV infection, accounting for 20% of cases in regions endemic for HEV<sup>[4]</sup>. Importantly, such liver failure caused by HBV and HEV results in high mortality rates. However, there are still no data on anti-HBV treatment for liver failure caused by the superinfection of HBV and HEV, as previous studies did not consider

patients with superinfection.

The aim of our study was to investigate the impact of chronic HBV infection on superimposed acute hepatitis E, particularly the influence of the status of HBeAg, HBV DNA, and cirrhosis on disease severity. Furthermore, we evaluated the effect of anti-HBV treatment on HBV-HEV superinfection. The use of a single liver function index is limited in assessing liver function, but the Model for End-Stage Liver Disease (MELD) score<sup>[14]</sup>, which combines multiple indices, can play a useful role in this assessment. The MELD score system has been used extensively for the allocation of donor livers worldwide<sup>[15]</sup> and has been validated for use in chronic hepatitis B (CHB)<sup>[16]</sup>. Thus, the MELD score was applied for a comprehensive analysis of liver function.

## MATERIALS AND METHODS

### Patients

This work was approved by the local ethics committee of our university. A total of 294 patients were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University, from January 2003 to January 2012. Among these patients, 118 were diagnosed with acute hepatitis E and chronic HBV superinfection (HBV + HEV group), and 176 patients were diagnosed with acute hepatitis E alone (HEV group). Acute hepatitis E was diagnosed when patients were hospitalized with typical symptoms of acute viral hepatitis and the presence of anti-HEV serum IgM and IgG. The presence of HBsAg and the absence of anti-HBc IgM established a diagnosis of chronic HBV infection. The diagnosis of liver failure was based on the Guidelines for Diagnosis of Liver Failure (2006)<sup>[17]</sup> and included the presence of two or more of the following: an international normalized ratio (INR)  $\geq 1.5$ , serum total bilirubin (TBil)  $> 10$  times the upper limit of normal, ascites, hepatic encephalopathy, decreased liver size, or hepatorenal syndrome. The complications that were observed were ascites, peritonitis, hepatic encephalopathy, gastrointestinal bleeding, and hepatorenal syndrome.

### Detection

Anti-HEV serum IgM and IgG were detected with an enzyme-linked immunosorbent assay (Genelabs Technologies, Singapore). HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb were detected with an automatic rapid immunoassay system (AxSYM; Abbott, United States). HBV DNA levels were determined by real-time polymerase chain reactions using commercial diagnostic kits (Da-an GeneCo., Guangzhou, China) with a lower detection limit of 500 copies/mL. Liver function tests were performed using an automatic biochemical analyzer (AU 640; Olympus, Japan). In this study, prothrombin time (PT)-INR (PT/reference PT) = international sensitivity index. The PT was measured using the detection reagent STA-Neoplastine(r) CI PLUS with an automatic coagulometer (STA-R) (Diagnostica Stago, France). A diagnosis of underlying cirrhosis was made based on clinical,

**Table 1** Demographic and clinical characteristics of the hepatitis B virus + hepatitis E virus group and hepatitis E virus group

	HBV + HEV group ( <i>n</i> = 118)	HEV group ( <i>n</i> = 176)	<i>P</i> values
Age (yr)	44.5 ± 13.8	54.1 ± 15.8	0.000 <sup>1</sup>
Sex			0.000 <sup>1</sup>
Male	109 (92.4)	140 (79.5)	
Female	9 (7.6)	36 (20.5)	
ALT (U/L)	266.0 ± 227.3	262.9 ± 212.9	0.905
AST (U/L)	228.9 ± 207.1	228.4 ± 213.1	0.986
PT (s)	22.1 ± 11.3	16.8 ± 7.9	0.000 <sup>1</sup>
PTA (%)	57.3 ± 26.6	77.2 ± 24.9	0.000 <sup>1</sup>
TBil (μmol/L)	334.7 ± 228.0	277.5 ± 217.4	0.031 <sup>1</sup>
MELD score	20.0 ± 9.7	15.1 ± 8.6	0.000 <sup>1</sup>
Complications	47 (39.8)	29 (16.5)	0.000 <sup>1</sup>
Liver failure	42 (35.6)	15 (8.5)	0.000 <sup>1</sup>
Death	24 (20.3)	13 (7.4)	0.002 <sup>1</sup>

<sup>1</sup>Denotes significant *P* value. Data are expressed as absolute *n* (%) or mean ± SD. HBV: Hepatitis B virus; HEV: Hepatitis E virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; PTA: Prothrombin activity; TBil: Serum total bilirubin; MELD: Model for End-Stage Liver Disease.

biochemical, and ultrasonographic findings. Sample collection, transportation, preservation, and processing were performed according to the manufacturer's instructions.

#### Calculation of MELD scores

MELD score =  $3.8 \times \log_e$  [serum bilirubin (μmol/L) × 0.058] +  $11.2 \times \log_e$  (PT-INR) +  $9.6 \times \log_e$  [serum Cr (μmol/L) × 0.011] + 6.4 × (0 or 1) (cholestatic or alcoholic cirrhosis: 0; other liver diseases: 1)<sup>[14]</sup>.

#### Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, United States). The  $\chi^2$  test or Fisher's exact test were used for categorical variables, and the Student's *t* test was used for continuous variables. Continuous variables are expressed as the mean ± SD, and categorical variables are expressed as the percentage (number). *P* values < 0.05 were considered statistically significant.

## RESULTS

### Demographic characteristics

The demographic characteristics of the 294 patients are shown in Table 1. The males outnumbered the females in both groups. The mean ages at admission were 44.5 and 54.1 years in the HBV + HEV and HEV groups, respectively.

### Laboratory findings

Liver function tests were performed at admission and regularly after admission. We compared the most severe laboratory abnormalities in the biochemical profile between the two groups (Table 1). The mean peak values of PT (22.1 *s* *vs* 16.8 *s*, *P* = 0.000) and TBil (334.7 μmol/L *vs* 277.5 μmol/L, *P* = 0.031), as well as the mean MELD

score (20.0 *vs* 15.1, *P* = 0.000), were significantly higher in the HBV + HEV group compared with the HEV group. In contrast, the mean values of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) did not differ significantly between the two groups.

### Clinical outcomes

As shown in Table 1, the incidences of complications, liver failure, and death were compared between the two groups with respect to clinical outcomes. Complications were noted in 39.8% (47/118) and 16.5% (29/176) of the patients in the HBV + HEV and HEV groups, respectively, and the occurrence of complications in the HBV + HEV group was significantly higher than that in the HEV group (*P* = 0.000). The incidence of liver failure was significantly higher among patients with superinfection than among patients with acute hepatitis E alone (35.6% *vs* 8.5%, *P* = 0.000). The mortality rates were also significantly different between the two groups (20.3% in the HBV + HEV group and 7.4% in the HEV group).

### Influence of chronic status of HBV infection on acute hepatitis E

To evaluate the influence of chronic HBV infection (based on the status of HBeAg, HBV DNA, and cirrhosis), we performed further analysis of the HBV + HEV group (Table 2). Of the 118 patients in the HBV + HEV group, 16.9% (20/118) were HBeAg-positive, 55.1% (65/118) were HBV DNA-positive, and 14.4% (17/118) had underlying cirrhosis. The occurrence of complications, liver failure, and death did not differ significantly between the HBeAg (+/-) and HBV DNA (+/-) subgroups. Patients with underlying cirrhosis had a significantly higher incidence of complications and liver failure. The mortality rate was 23.5% in the cirrhosis subgroup and 19.8% in the non-cirrhosis subgroup, which was not a significant difference.

### Anti-HBV treatment in the HBV + HEV group

Of the 118 patients in the HBV + HEV group, only 15 (12.7%) patients took oral anti-HBV agents. Three of these patients received lamivudine, and the other 12 patients received entecavir. In the HBV + HEV group, 42 patients developed liver failure, and 28.6% received anti-HBV treatment. Only 3.9% of patients without liver failure received anti-HBV treatment.

The mean mortality rates among the 42 patients with liver failure were 66.7% and 53.3% for patients who were and were not receiving anti-HBV treatment, respectively, which were not significantly different (Table 3).

## DISCUSSION

Due to the high prevalence of both HBV and HEV infection and the lack of cross-immunity between the two viruses, HBV-HEV superinfection is common<sup>[3,18]</sup>.

In our study, patients with acute hepatitis E superimposed on chronic HBV infection had higher peak laboratory abnormalities and poorer outcomes. There was also

**Table 2** Influence of chronic hepatitis B virus infection on acute hepatitis E *n* (%)

	HBV + HEV group								
	Status of HBeAg			Status of HBV DNA			Status of cirrhosis		
	+	-	<i>P</i> value	+	-	<i>P</i> value	+	-	<i>P</i> value
MELD score	17.0 ± 7.0	20.6 ± 10.1	0.131	21.5 ± 10.6	18.3 ± 8.3	0.074	24.7 ± 12.2	19.2 ± 9.1	0.03 <sup>1</sup>
Liver failure	5 (25)	37 (37.8)	0.317	25 (38.5)	17 (32.1)	0.563	11 (64.7)	31 (30.7)	0.01 <sup>1</sup>
Complications	7 (35)	40 (40.8)	0.803	25 (38.5)	22 (41.5)	0.850	16 (94.1)	31 (30.7)	0.00 <sup>1</sup>
Death	2 (10)	22 (22.4)	0.359	14 (21.5)	10 (19.9)	0.820	4 (23.5)	20 (19.8)	0.748

<sup>1</sup>Denotes significant *P* value. HBV: Hepatitis B virus; HEV: Hepatitis E virus; HBeAg: Hepatitis B e antigen; MELD: Model for End-Stage Liver Disease.

**Table 3** Anti-hepatitis B virus treatment in patients with liver failure in the hepatitis B virus + hepatitis E virus group

	Patients receiving anti-HBV treatment ( <i>n</i> = 12)	Patients not receiving anti-HBV treatment ( <i>n</i> = 30)	<i>P</i> value
HBV DNA (log <sub>10</sub> copies/mL)	4.99e7 ± 9.82e7	2.35e8 ± 9.20e8	0.495
MELD score	33.2 ± 9.4	28.3 ± 9.2	0.129
Mortality	8 (66.7)	53.3 (16/30)	0.506

Data are expressed as absolute *n* (%) or mean ± SD. HBV: Hepatitis B virus; HEV: Hepatitis E virus; MELD: Model for End-Stage Liver Disease.

a higher prevalence of liver failure among those patients. The present study confirmed the previous finding that acute HEV infection can cause severe liver injury in patients with chronic HBV infection<sup>[4,7]</sup>. This result indicates that chronic HBV infection has a negative impact on the clinical features of acute hepatitis E. However, it remains unknown whether the disease severity of acute hepatitis E correlates with the underlying HBV replication status or liver histological lesions. Our further analysis of the superinfection group showed that the disease severity of superimposed acute hepatitis E correlated not with the HBV replication status (based on the status of HBeAg and HBV DNA), but rather with the presence of underlying liver histological lesions (liver cirrhosis).

It has long been suggested that patients with chronic HBV infection are immunologically different from people without HBV infection. For instance, patients with chronic HBV infection have been reported to have impaired cell-mediated immunity<sup>[19,22]</sup>, decreased peripheral-blood T cell numbers<sup>[23,24]</sup>, impaired interferon production<sup>[25,26]</sup>, and imbalanced cytokine levels<sup>[27,28]</sup> and may have other currently unrecognized differences. The severity of acute viral hepatitis has been suggested to be dependent on host immune factors rather than on the direct toxicity of the virus. Thus, with impaired and imbalanced immunity in chronic HBV infection, HEV may trigger an excessive immunological response and then induce severe damage in hepatocytes. Alternatively, hepatocyte impairment may accumulate during the chronic course of HBV infection. Thus, with preexisting liver lesions, especially due to cirrhosis, hepatocytes may be limited in their ability to regenerate. This limitation contributes to more severe liver injury in patients with acute hepatitis E superimposed on chronic HBV infection.

According to our data, most patients in the HBV + HEV group were HBeAg-negative, and nearly 50% were HBV DNA-negative. Further analysis showed that the disease severity of acute hepatitis E did not correlate with the status of HBeAg or HBV DNA. This finding indicates that in the superinfection of HBV and HEV, chronic HBV infection is inactive, and HEV is the main trigger factor for severe disease. Thus, the finding raises the question of whether anti-HBV treatment improves the outcome of HBV-HEV superinfection, which requires further investigation.

Acute exacerbation frequently occurs in the natural course of chronic HBV infection. In the case of acute exacerbation caused by spontaneous HBV activation, anti-HBV treatment can strongly suppress HBV replication, and most patients can recover. However, certain patients may develop liver failure, which is named HBV-related acute-on-chronic liver failure (HBV-ACLF). HBV-ACLF remains a rapidly progressive and frequently fatal condition for which mortality reaches 25% to 35%. International guidelines recommend initiating nucleos(t)ide analogs as early as possible in this patient population<sup>[8,9]</sup>. Recent studies have shown that anti-HBV treatment can improve the outcome of HBV-ACLF<sup>[10-13]</sup>. Superinfection with HEV is another common cause of liver failure in chronic HBV infection and is present in 20% of cases in regions endemic for HEV<sup>[4]</sup>. Importantly, such liver failure caused by HBV and HEV results in high rates of mortality. However, for the liver failure caused by the superinfection of HBV and HEV, there are still no data on anti-HBV treatment. In our study, we evaluated the results of anti-HBV treatment administration to the HBV + HEV group. Of the 76 patients without liver failure, only 3.9% took anti-HBV drugs, but the prognosis of this patient population was good. This finding indicates that it is not necessary to administer anti-HBV treatment as soon as possible to patients with HBV-HEV superinfection in mild disease. The necessity of anti-HBV treatment for HBV infection should be re-evaluated by monitoring the HBV DNA level and liver function tests after recovery from acute hepatitis E.

As shown by our data, up to 28.6% of patients received anti-HBV treatment once superinfection caused liver failure. The mortality rate among patients receiving anti-HBV treatment was 66.7%, which was not significantly different from the mortality rate of patients not receiving anti-HBV treatment. Thus, anti-HBV treatment

was unable to improve the outcome of the liver failure caused by HBV-HEV superinfection. As mentioned previously, HEV infection plays the most important role in the disease. The infection triggers strong immunological injury in hepatocytes, which results in liver failure. Anti-HBV treatment can inhibit HBV replication but cannot stop the strong immune activity, so the therapy cannot improve the outcome of this patient population.

In conclusion, our study indicates that acute hepatitis E is associated with more severe disease in patients with chronic HBV infection and that disease severity correlates with the underlying cirrhosis in chronic HBV infection. Anti-HBV treatment cannot improve the prognosis of liver failure caused by HBV-HEV superinfection. As HBV vaccination is being aggressively pursued worldwide, HEV vaccination should also be considered in endemic areas when a vaccine becomes available<sup>[29]</sup>. Additionally, preventive measures are important to the related morbidity and mortality, such as the consumption of boiled water and well-cooked food.

The main limitation of the present study is its retrospective nature. HEV-RNA levels were not regularly checked, so we are not convinced that there is no substantial contribution of HEV load to the disease severity of HBV-HEV superinfection. Additionally, underlying cirrhosis was mainly assessed by ultrasonography. Thus, it is possible that early-stage underlying cirrhosis was missed. Despite these limitations, our study is significant because the work provides preliminary support for not administering anti-HBV treatment in HBV-HEV superinfection, as chronic HBV replication status (based on the status of HBeAg and HBV DNA) does not determine the outcome of HBV-HEV superinfection.

## COMMENTS

### Background

Hepatitis B virus (HBV)-hepatitis E virus (HEV) superinfection is common. Recent studies have shown that HEV can result in severe disease in patients with underlying chronic HBV infection and even liver failure. However, whether the disease severity of acute hepatitis E correlates with the underlying HBV replication status (based on the status of HBeAg and HBV DNA) or liver histological lesions and whether anti-HBV treatment can improve the outcome of HBV-HEV superinfection are unknown.

### Research frontiers

The present study investigated the influence of chronic HBV infection (based on the status of HBeAg, HBV DNA, and cirrhosis) on superimposed acute hepatitis E. Furthermore, the study evaluated the effect of anti-HBV treatment on HBV + HEV superinfection.

### Innovations and breakthroughs

The disease severity of superimposed acute hepatitis E correlated not with the HBV replication status (based on the status of HBeAg and HBV DNA), but rather with the presence of underlying cirrhosis. Anti-HBV treatment did not improve the prognosis of liver failure caused by HBV-HEV superinfection.

### Peer review

This study is of great interest and offers useful implications for the treatment of HBV-HEV superinfection.

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## A phase II study of paclitaxel and nedaplatin as front-line chemotherapy in Chinese patients with metastatic esophageal squamous cell carcinoma

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### Abstract

**AIM:** To evaluate the efficacy and safety of paclitaxel-nedaplatin combination as a front-line regimen in Chinese patients with metastatic esophageal squamous cell carcinoma (ESCC).

**METHODS:** A two-center, open-label, single-arm phase II study was designed. Thirty-nine patients were enrolled and included in the intention-to-treat analysis of efficacy and adverse events. Patients received 175 mg/m<sup>2</sup> of paclitaxel over a 3 h infusion on 1 d, followed by nedaplatin 80 mg/m<sup>2</sup> in a 1 h infusion on 2 d every 3 wk until the documented disease progression, unac-

ceptable toxicity or patient's refusal.

**RESULTS:** Of the 36 patients assessable for efficacy, there were 2 patients (5.1%) with complete response and 16 patients (41.0%) with partial response, giving an overall response rate of 46.1%. The median progression-free survival and median overall survival for all patients were 7.1 mo (95%CI: 4.6-9.7) and 12.4 mo (95%CI: 9.5-15.3), respectively. Toxicities were moderate and manageable. Grade 3/4 toxicities included neutropenia (15.4%), nausea (10.3%), anemia (7.7%), thrombocytopenia (5.1%), vomiting (5.1%) and neutropenia fever (2.6%).

**CONCLUSION:** The combination of paclitaxel and nedaplatin is active and well tolerated as a first-line therapy for patients with metastatic ESCC.

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**Key words:** Esophageal squamous cell cancer; Front-line chemotherapy; Paclitaxel; Nedaplatin

**Core tip:** Esophageal cancers are among the most aggressive tumors with a poor prognosis. Till now, there has been no standard chemotherapy regimen for advanced esophageal cancer. In this paper, we conducted a phase II study on combination chemotherapy consisting of paclitaxel and nedaplatin in previously untreated patients with metastatic esophageal squamous cell carcinoma (ESCC). Our results demonstrated that the combination of two drugs is active and well tolerated as a first-line therapy for patients with recurrent or metastatic ESCC.

He YF, Ji CS, Hu B, Fan PS, Hu CL, Jiang FS, Chen J, Zhu L, Yao YW, Wang W. A phase II study of paclitaxel and nedaplatin

as front-line chemotherapy in Chinese patients with metastatic esophageal squamous cell carcinoma. *World J Gastroenterol* 2013; 19(35): 5910-5916 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i35/5910.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5910>

## INTRODUCTION

China accounts for about half of the world's esophageal cancer cases, about 250000 each year, and 85% of the total global incidence occurs in the developing world, according to the World Health Organization report. Overt and incurable metastatic disease is present at diagnosis in 50% of patients. Furthermore, even after curative surgery, local recurrences and/or distant metastases are detected in more than 50% of the patients within 5 years of follow-up<sup>[1]</sup>. The median survival of patients with metastatic esophageal carcinoma is only 3-8 mo<sup>[2]</sup>. Palliative chemotherapy may lead to distant tumor and symptom control. The effect of chemotherapy on survival is unclear for lack of large randomized trials. Up till now there has been no global standard regimen for the first-line treatment of advanced disease. Of the available regimens, the regimen containing 5-fluorouracil (5-FU) and cisplatin is widely used in China, with RR ranging from 15%-45%<sup>[3-5]</sup>. However, treatment with 5-FU and cisplatin can induce severe toxicity<sup>[6]</sup>. What's more, almost all patients have to be hospitalized for this treatment. Therefore, it is imperative to develop effective and well-tolerated chemotherapeutic agents for treatment.

Recently, paclitaxel, a natural product isolated from the bark of the yew tree *Taxus brevifolia*, has demonstrated some promising responses against digestive tract cancer. As a single agent, paclitaxel has been reported to achieve a response rate of 32% in esophageal cancer and gastroesophageal junction cancer<sup>[7]</sup>. Besides, several phase I / II studies have shown that paclitaxel-based regimens have significant activity in patients with locally advanced and metastatic esophageal cancer<sup>[8-12]</sup>. However, toxicity for combination therapy was significant and included severe myelosuppression, gastrointestinal (GI) and neurologic toxicity, and a significant rate of hospitalization for treatment-related complications. So it is urgent to seek new combination treatments that could achieve similar outcome and induce relatively minimal toxicities.

Nedaplatin cis-diammine-glycolate platinum (NDP) is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclinical antitumor activity against various solid tumors with lower nephrotoxicity<sup>[13]</sup>. Preclinical studies indicate that nedaplatin has an antitumor activity comparable to cisplatin<sup>[14-16]</sup> and has been shown experimentally to overcome cisplatin resistance in a cisplatin-resistant K562 cell line<sup>[14]</sup>. Clinically, single agent nedaplatin has shown a wide spectrum of antitumor activity, producing the favorable response rates in head and neck<sup>[17]</sup>, esophagus<sup>[18]</sup>, non-small cell lung<sup>[19,20]</sup>, and cervical cancers<sup>[21]</sup>. These reports prompted us to use

a new combination of nedaplatin and paclitaxel for patients with metastatic esophageal carcinoma, because these patients have poorer tolerance, and a less toxic treatment is desirable. The current phase II study was conducted to evaluate the efficacy and safety of nedaplatin-paclitaxel combination as a front-line regimen in Chinese patients with metastatic esophageal squamous cell carcinoma.

## MATERIALS AND METHODS

### Study design

This was a two-center, open-label, single-arm phase II study evaluating the efficacy and toxicities of nedaplatin and paclitaxel in patients with metastatic esophageal squamous cell carcinoma who had no previous treatment. The primary end point was response to treatment. Secondary end points were toxicity, progression-free survival (PFS) and overall survival (OS).

### Eligibility criteria

Patients aged 18-75 years with measurable target lesion pathologically confirmed advanced or metastatic esophageal squamous cell carcinoma were eligible for the study. Prior chemotherapy for advanced disease was not permitted. However, neoadjuvant or concurrent chemotherapy was allowed, provided that the treatment was completed at least 6 mo before the start of the current study. Patients were required to have Eastern Cooperative Oncology Group (ECOG) performance status of 0-2, with a life expectancy  $\geq 3$  mo, an adequate bone marrow, liver and kidney function, as indicated by an absolute neutrophil count (ANC)  $\geq 1500/\mu\text{L}$ , a platelet count  $\geq 100000/\mu\text{L}$ , serum creatinine  $\leq 2.0$  mg/dL, serum bilirubin  $\leq 1.5$  mg/dL, and serum alanine aminotransferase  $\leq 2.5$  times higher than the upper limit of the normal (except in those cases with liver involvement when a value  $\leq 5$  times the upper limit of the normal was accepted). All patients were given written informed consents to participate in this study, which was also approved by the Ethics Committee of two centers.

### Exclusion criteria

Patients with evidence of central nervous system metastases, an inability to take oral medication were excluded. Gastroesophageal junction tumors were excluded from the study. Exclusion criteria also included pathologically confirmed adenocarcinoma, prior malignancies (other than non-melanoma skin cancer or *in situ* cervical cancer) within the previous 5 years, and uncontrolled infection or severe comorbidity such as myocardial infarction within 6 mo or symptomatic heart diseases. Pregnant or lactating women were excluded from the study; women with childbearing potential were required to agree to have adequate contraception.

### Study treatment

Patients received 175 mg/m<sup>2</sup> of paclitaxel over a 3 h infusion on 1 d, followed by nedaplatin 80 mg/m<sup>2</sup> in a 1 h

infusion on 2 d every 3 wk until the documented disease progression, unacceptable toxicity or patient's refusal. These doses were based on a phase I trial of chemotherapy using paclitaxel and nedaplatin in chemotherapy-naïve patients with unresectable squamous cell carcinoma (SCC)<sup>[22]</sup>. Paclitaxel infusions preceded the administration of nedaplatin in the current study, as the interaction of nedaplatin and paclitaxel is highly schedule-dependent<sup>[23,24]</sup>. As prophylactic agents, dexamethasone (*iv* 20 mg), promethazine (*iv* 25 mg) and cimetidine (*iv* 400 mg) were given 30 min before paclitaxel administration. All patients received adequate antiemetic therapy prior to chemotherapy. Granulocyte colony-stimulating factor was administered at physician's discretion.

### Response of treatment and adverse effects

All patients were screened for medical history and underwent a physical examination. Complete blood cell count (CBC) was performed every week, blood biochemical test and electrocardiogram were performed before every cycle. After every two cycles of treatment, response was evaluated by two independent experts using RECIST criteria. Of the lesions observed prior to treatment, a maximum of five measurable lesions from each metastasized organ up to a total of 10 lesions were selected as target lesions. In the cases of partial response (PR) or complete response (CR), a confirmative computed tomography (CT) scan was performed 4 wk later and this was followed by a CT scan after every two treatment cycles. After discontinuation of treatment, follow-up visits were done every 3 mo to document late toxic effects, disease progression and survival. Toxicity was reported using an NCI-CTC version 3.0 toxicity scale.

### Dose modification

The dose of paclitaxel was reduced to 150 mg/m<sup>2</sup> if one of the following conditions occurred: grade 3 neutropenia with infection, grade 4 neutropenia, grade 3 thrombocytopenia or > grade 3 sensory neurotoxicity. If toxicity persisted, a second dose reduction of paclitaxel to 135 mg/m<sup>2</sup> was allowed. In cases of fatigue or asthenia above grade 3, treatment was postponed for 1 wk and restarted when the patient recovered to below grade 2. Patients requiring a delay in therapy for > 2 wk or more than two dose reductions were removed from the study. A new cycle of therapy could begin if the neutrophils count were  $1.5 \times 10^9/L$ , the platelets count were  $75 \times 10^9/L$ , and all relevant nonhematological toxicities were grade 2. Once a dose had been reduced during a treatment cycle, re-escalation was not permitted during any other subsequent cycles.

### Statistical analysis

A Simon's two stage phase II design was used. The treatment program was designed to reject response rates of 20% and to provide a significance level of 0.05 with a statistical power of 80% to assess the activity of the regimen at a 40% response rate<sup>[25]</sup>. The upper limit for a first-stage treatment rejection was 4 responses among 18 evaluable patients; the upper limit of second-stage rejection was 10

responses among 33 evaluable patients. Assuming a dropout rate of 20%, a total of 39 patients were required. All enrolled patients were included in the intention-to-treat (ITT) analysis of efficacy. Analysis of PFS and overall survival analysis were performed by the Kaplan-Meier method. The PFS was calculated from the initiation of chemotherapy to the date of the disease progression, while overall survival was measured from the initiation of chemotherapy to the date of the last follow-up or death. Statistical data were obtained using an SPSS 11.0 software package (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Patients' characteristics

Between June 2008 and July 2010, a total of 39 patients from 2 centers (Including Anhui Provincial Hospital affiliated to Anhui Medical University and Anhui Provincial Cancer Hospital) were enrolled. Their baseline characteristics are shown in Table 1.

### Efficacy and survival

A total of 39 patients were assessable for response (Table 2). Two patients were not evaluated because of loss to follow-up after two courses, and 1 patient withdrew consent because of toxicities after 1 course. Two patients (5.1%) achieved a CR, 16 patients (41.0%) had PR, 15 patients (38.5%) had SD and 3 patients had PD (7.7%). The median follow-up period was 13.1 mo (range 3.3-28.6 mo). The median PFS for all patients was 7.1 mo (95%CI: 4.6-9.7, Figure 1A). The median OS was 12.4 mo (95%CI: 9.5-15.3, Figure 1B), with a 1-year survival rate of 53.8%.

### Adverse events

A total of 141 courses of treatment were given and patients received a median 4 courses (range, 1-6 courses). AE frequencies in this population are listed in Table 3. The most common haematologic AE was leucopenia, which occurred with grade 3/4 in 6 patients (15.4%). Febrile neutropenia was observed in 1 patient (2.6%). Although this case was successfully treated with antibiotics and G-CSF, this patient withdrew his consent after this experience. Grade 3/4 anemia was observed in 3 patients (7.7%) and grade 3 thrombocytopenia in 2 patients (5.1%). Major nonhematologic AEs (in order of decreasing frequency) were nausea (59.0%), fatigue (56.4%), vomiting (46.2%), myalgia (43.6%), alopecia (30.8%), and diarrhea (15.4%). Grade 3/4 nausea vomiting was observed in 4 patients (10.3%) and in 2 patients (5.1%). Hepatic and renal toxicities were mild. No treatment-related death occurred during this study.

## DISCUSSION

Esophageal cancer has two main pathological forms: squamous cell carcinoma (ESCC) and adenocarcinoma. Because cardiac adenocarcinoma has been usually classified as gastric cancer, primary esophageal adenocarci-

**Table 1** Patients' characteristics (*n* = 39) *n* (%)

Characteristics	No. of patients
Age, yr (range)	Median 60 (range, 34-72)
Sex	
Female	1 (2.6)
Male	38 (97.4)
ECOG performance status	
0	4 (10.3)
1	32 (82.0)
2	3 (7.7)
Tumor involved site	
Lymph node	25 (64.1)
Lung	14 (35.9)
Liver	16 (41.0)
Bone	7 (17.9)
Number of involved site	
1	28 (71.8)
2	13 (33.3)
≥ 3	4 (10.3)
Differentiation	
Poor-differentiated	10 (25.6)
Moderate-well differentiated	23 (59.0)
Unknown	6 (15.4)
Prior treatment (cases)	
Treatment-naive	30 (76.9)
Radiation	4 (10.3)
Operation	5 (12.8)

ECOG: Eastern Cooperative Oncology Group.

noma represents only < 1% of esophageal cancer patients in China<sup>[26]</sup>. In view of most cases being ESCC, we focused on this type of esophageal cancer in our study. Recurrent or metastatic ESCC remains incurable disease. Systematic combined chemotherapy has been part of combined modality therapy as a palliative treatment for this patient population.

However, there is no standard chemotherapy regimen for advanced esophageal cancer, various kinds of chemotherapy regimens have been tried to prolong survival and improve quality of life. The most commonly used regimen as the first-line chemotherapy is the combination of cisplatin (100 mg/m<sup>2</sup> per day) and 5-FU (1000 mg/m<sup>2</sup> per day continuous infusion for 96-120 h) in metastatic esophageal cancer<sup>[27]</sup>. The randomized phase II study comparing cisplatin/5-FU to cisplatin alone in advanced squamous cell esophageal cancer demonstrated that the combination arm was superior to cisplatin alone arm in terms of RR (35% *vs* 19%, respectively), and OS (33 wk *vs* 28 wk, respectively)<sup>[6]</sup>. However, high rate of treatment-related deaths (16%) was not acceptable. What's more, continuous infusion of 5-FU requires an indwelling venous access, which provides a source for venous thrombosis and sepsis and makes therapy burdensome to the patient. Until recently, newer agents such as taxanes (paclitaxel and docetaxel), vinorelbine, irinotecan, capecitabine, oxaliplatin and nedaplatin have been investigated as single agent or in combination in neoadjuvant or palliative settings<sup>[28]</sup>.

In the current study, the overall RR was 46.2% (50.0% for 36 valuable patients), the disease control rate was

**Table 2** Tumor response (intention-to-treat analysis) *n* (%)

Response	<i>n</i> = 39
Response rate	
Complete response	2 (5.1)
Partial response	16 (41.0)
Stable disease	15 (38.5)
Progressive disease	3 (7.7)
Not assessable	3 (7.7)

84.6% with a median TTP of 7.1 mo and a median OS of 12.4 mo. This study shows that this regimen has encouraging antitumor activity. Recently, several phase II studies were published of paclitaxel and platinum based regimens for advanced or metastatic esophageal cancer<sup>[5,8,9,11,12]</sup>. Gong *et al*<sup>[5]</sup> reported that the overall RR was 43.6% and the median progression-free survival (PFS) and OS was 6 and 10 mo, respectively, in a phase II study with metastatic esophageal cancer treated with the same combination regimen. Polee *et al*<sup>[11]</sup> reported that paclitaxel and cisplatin induced a relative longer median PFS of 8 mo, but the median time of OS was only 9 mo. The highest median OS (13 mo) was reported with 7 mo of median TTP by Zhang *et al*<sup>[12]</sup>. The results of our study can be consistent with those of these published studies.

In consideration of the performance status and chemotherapy tolerance of cancer patients in metastatic setting, treatment related toxicities should be strictly limited. In the present study, the most common grade 3/4 toxicities were leucopenia (15.4%), nausea (10.3%) and anemia (7.7%), thrombocytopenia (5.1%), vomiting (5.1%), respectively. Only one patient with febrile neutropenia was discontinued from the study, who was successfully treated with antibiotics and G-CSF. There was no treatment-related death during this study. The toxicities of nedaplatin and paclitaxel regimen were similar with the paclitaxel based regimen reported by Zhang *et al*<sup>[12]</sup> and Ilson *et al*<sup>[10]</sup>, and more minimal than other studies which applied gemcitabine plus cisplatin, paclitaxel plus carboplatin, nedaplatin plus docetaxel, or irinotecan plus cisplatin/cisplatin-5-FU<sup>[9,29-33]</sup>. The combination of nedaplatin and paclitaxel was deemed safe in patients with metastatic esophageal carcinoma in spite of the observed toxicity.

So far, some drugs have been applied for advanced esophageal carcinoma, such as capecitabine and oxaliplatin. More recently, a randomized phase III trial evaluated capecitabine and oxaliplatin as alternatives to infused 5-FU and cisplatin, respectively, for untreated advanced esophagogastric carcinoma<sup>[34]</sup>. The more active regimens including epirubicin, oxaliplatin and capecitabine achieved the median PFS of 7 mo and median OS of 11.2 mo, while the relative higher treatment-related toxicities were reported. Of note, all the patients in that study were pathologically confirmed adenocarcinoma. So, the standard chemotherapy for advanced or metastatic ESCC still needs more clinical trials.

In conclusion, the results from our phase II study demonstrated that the combination of nedaplatin and

**Table 3 Adverse events assessment n (%)**

Adverse event	NCI-CTC grade (n = 39)				Any	Grade 3/4
	1	2	3	4		
<b>Hematologic</b>						
Leucopenia	15 (25.6)	13 (33.3)	4 (10.3)	2 (5.1)	34 (87.2)	15.4%
Anemia	17 (43.6)	5 (12.8)	2 (5.1)	1 (2.6)	25 (64.1)	7.7%
Thrombocytopenia	11 (28.2)	5 (12.8)	2 (5.1)	0 (0.0)	18 (46.2)	5.1%
<b>Nonhematologic</b>						
<b>Gastrointestinal</b>						
Nausea	11 (28.2)	8 (20.5)	4 (10.3)	0 (0.0)	23 (59.0)	10.3%
Vomiting	10 (25.6)	6 (15.4)	2 (5.1)	0 (0.0)	18 (46.2)	5.1%
Diarrhea	6 (15.4)	0 (0.0)	0 (0.0)	0 (0.0)	6 (15.4)	0.0%
Stomatitis	2 (5.1)	1 (2.6)	0 (0.0)	0 (0.0)	3 (7.7)	0.0%
<b>Hepatic</b>						
AST	2 (5.1)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.1)	0.0%
ALT	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.6)	0.0%
<b>Renal</b>						
Serum creatine	2 (5.1)	0	0 (0.0)	0 (0.0)	2 (5.1)	0.0%
Alopecia	3 (7.7)	9 (23.1)	0 (0.0)	0 (0.0)	12 (30.8)	0.0%
Myalgia	12 (30.8)	5 (12.8)	0 (0.0)	0 (0.0)	18 (43.6)	0.0%
Fatigue	20 (51.3)	2 (5.1)	0 (0.0)	0 (0.0)	22 (56.4)	0.0%
Neutropenia fever	0 (0.0)	0 (0.0)	1 (2.6)	0 (0.0)	1 (2.6)	2.6%

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

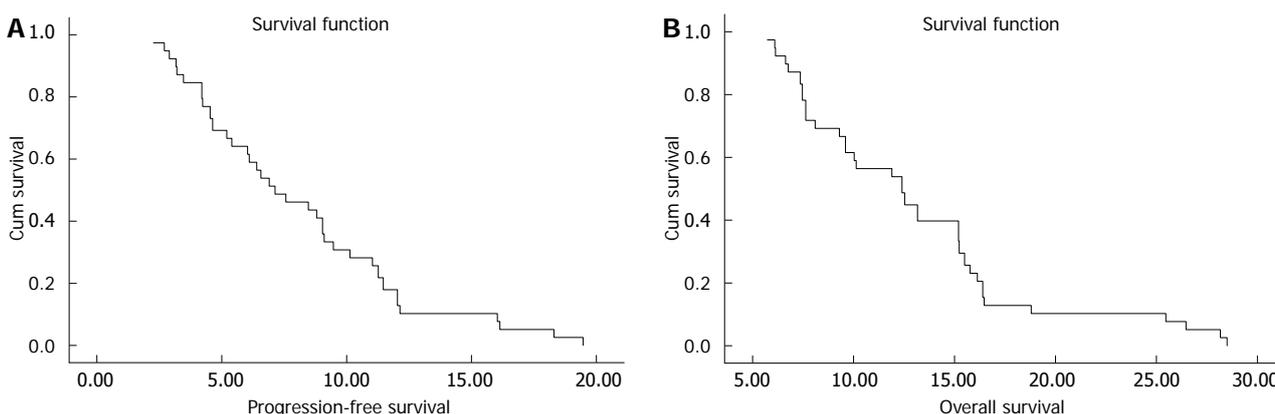


Figure 1 Kaplan-Meier analysis of progression-free survival (A) and overall survival (B) in the study population

paclitaxel is active and well tolerated as a first-line therapy for patients with recurrent or metastatic ESCC. It provides recurrent or metastatic ESCC patients with an effective, safe and convenient chemotherapeutic strategy.

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### COMMENTS

#### Background

Esophageal cancers are among the most aggressive tumors with a poor prognosis. Till now, there has been no standard chemotherapy regimen for advanced esophageal cancer. In this paper, the author conducted a phase II study on combination chemotherapy consisting of paclitaxel and nedaplatin in previously untreated patients with metastatic esophageal squamous cell carcinoma (ESCC).

#### Research frontiers

The most commonly used regimen as the first-line chemotherapy is the combi-

nation of cisplatin (100 mg/m<sup>2</sup> per day) and 5-fluorouracil (1000 mg/m<sup>2</sup> per day continuous infusion for 96-120 h) in metastatic esophageal cancer. However, high rate of treatment-related deaths (16%) was not acceptable. So, new regimens were explored to improve the efficacy and safety in metastatic ESCC.

#### Innovations and breakthroughs

The results demonstrated that the combination of nedaplatin and paclitaxel is active and well tolerated as a first-line therapy for patients with recurrent or metastatic ESCC. It provides recurrent or metastatic ESCC patients with an effective, safe and convenient chemotherapeutic strategy.

#### Applications

The combination of paclitaxel and nedaplatin is active and well tolerated as a first-line therapy for patients with metastatic ESCC.

#### Terminology

Paclitaxel, a natural product isolated from the bark of the yew tree *Taxus brevifolia*, has demonstrated some promising responses against digestive tract cancer. And nedaplatin is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclinical antitumor activity against various solid tumors with lower nephrotoxicity.

#### Peer review

This is a good clinical study in which the authors evaluated the efficacy and safety of paclitaxel-nedaplatin combination as a front-line regimen in Chinese patients with metastatic ESCC. The results are interesting and suggest that the combination of the above two drugs is active and well tolerated as a first-line

therapy for patients with metastatic ESCC.

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## Association between vitamin D and hepatitis C virus infection: A meta-analysis

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### Abstract

**AIM:** To evaluate the association between 25-hydroxyvitamin D [25(OH)D] and sustained virological response (SVR) in hepatitis C virus (HCV) infected individuals.

**METHODS:** Relevant studies were identified by systematically searching MEDLINE databases up to March 2012 and abstracts of the European and American Congress of Hepatology conducted in 2011. Studies must provide information on SVR and the levels of 25(OH)D<sub>3</sub> and/or 25(OH)D<sub>2</sub> [henceforth referred to as 25(OH)D] in sera samples from HCV infected individuals. The inclusion criteria were: clinical studies that included HCV infected patients aged older than 18 years regardless of HCV genotype or ethnic group; provided information on SVR rates; and were reported in the English language

as full papers. Due to the heterogeneity of studies in categorizing serum vitamin D levels, a cut-off value of 30 ng/mL of serum 25(OH)D was used. Heterogeneity was assessed using  $I^2$  statistics. The summary odds ratios with their corresponding 95%CI were calculated based on a random-effects model.

**RESULTS:** Overall, 11 studies (8 observational and 3 interventional) involving 1575 individuals were included and 1117 HCV infected individuals (71%) showed low vitamin D levels. Most of the studies included mono-infected HCV individuals with the mean age ranging from 38 to 56 years. Four studies were conducted in human immunodeficiency virus/HCV infected individuals. Regarding vitamin D measurement, most of the studies employed radioimmunoassays ( $n = 5$ ) followed by chemiluminescence ( $n = 4$ ) and just one study employed high performance/pressure liquid chromatography (HPLC). Basal vitamin D levels varied from 17 to 43 ng/mL in the studies selected, and most of the HCV infected individuals had genotype 1 (1068/1575) with mean viral load varying from log 4.5-5.9 UI/mL. With regard to HCV treatment, most of the studies ( $n = 8$ ) included HCV individuals without previous treatment, where the pooled SVR rate was 46.4%. High rates of SVR were observed in HCV individuals with vitamin D levels above 30 ng/mL (OR = 1.57; 95%CI: 1.12-2.2) and those supplemented with vitamin D (OR = 4.59; 95%CI: 1.67-12.63) regardless of genotype.

**CONCLUSION:** Our results demonstrated high prevalence of vitamin D deficiency and high SVR in individuals with higher serum vitamin D levels or receiving vitamin D supplementation.

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**Key words:** Vitamin D; Hepatitis C; Therapy; Meta-analysis; Sustained virological response

**Core tip:** High vitamin D levels (above 30 ng/mL) or

supplementation are associated with sustained virological response in hepatitis C virus infected individuals.

Villar LM, Del Campo JA, Ranchal I, Lampe E, Romero-Gomez M. Association between vitamin D and hepatitis C virus infection: A meta-analysis. *World J Gastroenterol* 2013; 19(35): 5917-5924 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5917.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5917>

## INTRODUCTION

Viral hepatitis C is a serious public health problem worldwide infecting more than 130 million individuals<sup>[1]</sup>. Treatment of hepatitis C virus (HCV) infection is usually carried out using pegylated interferon (PEG-IFN) and ribavirin (RBV) for 24 wk for HCV genotypes 2 or 3, or 48 wk for HCV genotype 1 and the main objective of HCV therapy is a sustained virologic response (SVR), defined as an undetectable serum HCV-RNA level at 24 wk after the end of therapy. Rates of SVR range from 60%-70% in chronic hepatitis C (CHC) patients with genotypes 2 and 3, but is less than 50% in patients with genotype 1 using conventional therapy<sup>[2]</sup>.

Recently, studies were conducted to analyze the influence of genetic and metabolic factors in antiviral response<sup>[3-5]</sup>, and a recent review showed that vitamin D levels can influence HCV treatment<sup>[6]</sup>. Vitamin D itself is considered biologically inactive and is hydroxylated to 25-hydroxyvitamin D [25(OH)D] in the liver. 25(OH)D is the main circulating vitamin D metabolite and is used for classification of the vitamin D status<sup>[7,8]</sup>. In the kidney, 25(OH)D is converted to 1,25-dihydroxyvitamin D [1,25(OH)D] by 1-alpha-hydroxylase, however, it has been demonstrated that this conversion can occur in many extra-renal tissues including the liver<sup>[7,9]</sup>. Finally, 25(OH)D or 1,25(OH)<sub>2</sub>D bind to the ubiquitously expressed vitamin D receptor (VDR), which regulates approximately 3% of the human genome<sup>[10]</sup>. In this context, vitamin D deficiency has been associated with an increased risk of cancer<sup>[7,11]</sup>, cardiovascular<sup>[12,13]</sup>, autoimmune<sup>[14,15]</sup> and infectious diseases<sup>[6,16]</sup>.

Due to these facts, there is great research interest in the role of vitamin D status in various infectious diseases. Some studies have shown that high levels of serum vitamin D level are an independent predictor of SVR following anti-viral therapy, and higher SVR is achieved with vitamin D supplementation in CHC individuals<sup>[17-22]</sup>. However, Lange *et al*<sup>[18]</sup> found that vitamin D deficiency was associated with a lower SVR rate only in CHC genotype 2/3 patients (treated with PEG-IFN and RBV for 24 wk), but not in CHC genotype 1 patients. Moreover, Jazwinski *et al*<sup>[23]</sup> found no association between vitamin D levels and SVR in 82 African American genotype 1 CHC-naïve patients, treated with PEG-IFN and RBV.

As vitamin D has an uncertain clinical value in HCV

infected individuals and taking into consideration the limitations of previous reviews, we conducted an updated systematic review and meta-analysis to comprehensively assess vitamin D deficiency regarding antiviral therapy and the influence of vitamin D supplementation on SVR.

## MATERIALS AND METHODS

### Identification of studies

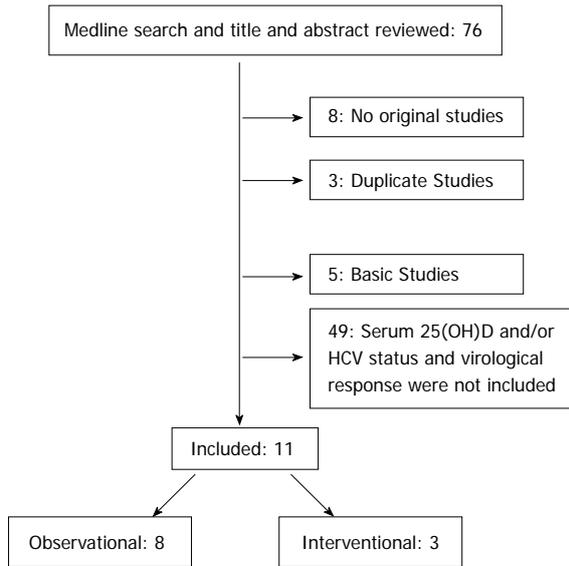
A broad search string was used in MEDLINE in order to identify relevant studies (all languages, all available years, search last completed 31.03.12) using the following search terms: [{"vitamin D"} [MeSH Terms] or {"vitamin D"} [All Fields] or {"ergocalciferols"} [MeSH Terms] or {"ergocalciferols"} [All Fields] or {"calcifediol"} [MeSH Terms] or {"calcifediol"} [All Fields] or {"calcidiol"} [All Fields] or {"25(OH)D"} [All Fields] or {"25(OH)D<sub>2</sub>"} [All Fields] or {"25(OH)D<sub>3</sub>"} [All Fields] and [{"HCV"} [MeSH Terms] or {"HCV"} [All Fields] or {"Hepacivirus"} [All Fields] or {"Hepacivirus"} [MeSH Terms]]. Abstracts from the European and American Association Congress of Hepatology (EASL 2011 and AASLD 2011) were also included in order to give more data on this theme.

Potentially relevant papers were accessed in order to review the abstract and/or full text. Only fully published articles were considered. Duplicate publications were deleted. Two researchers independently performed the literature search and data abstraction with regard to the inclusion and exclusion criteria by reading titles and abstracts. When reading titles and abstracts did not allow identification of eligible studies, articles were read in full. Only original studies conducted in humans were considered for the review. Thus, reviews and letters to the editor were excluded in the analysis, but read in full to identify potential relevant original studies. Disagreements between the two observers were resolved by discussion.

The following data were extracted: year of publication, number of patients, age, vitamin D levels, SVR percentage, method of measurement of vitamin D, HCV genotype, HCV viral load, percentage of naïve patients. When such data were not explicitly reported, they were derived from data provided in the articles or requested from the authors through personal contacts, wherever possible.

### Eligibility criteria

The study must provide information on SVR against HCV and the levels of 25(OH)D<sub>3</sub> and/or 25(OH)D<sub>2</sub> [henceforth referred to as 25(OH)D] in sera samples from HCV infected individuals. The inclusion criteria were: clinical studies that included HCV infected patients aged older than 18 years regardless of HCV genotype or ethnic group; provided information on SVR rates; and were reported in the English language as full papers. Studies were excluded if they met the following criteria: they did not provide information on 25(OH)D level, HCV status and/or SVR; basic studies; letters / case reports, or articles not reporting outcomes of interest or



**Figure 1** Prisma flowchart for the selection of publications for the systematic review and meta-analysis. HCV: Hepatitis C virus; 25(OH)D: 25-hydroxyvitamin D.

primary data (editorials, reviews).

### Statistical analysis

Data were extracted from each paper and compiled for hypovitaminosis D and HCV antiviral response. Statistical analysis was performed using the Meta-Disc software 1.4<sup>[24]</sup>, considering: (1) a summary of data from individual studies; (2) an investigation of the homogeneity of the studies both graphically and statistically; (3) calculation of clustered indices; and (4) exploration of heterogeneity. The meta-analysis was performed using the random-effect model by the Der Simonian and Laird method. Heterogeneity was tested for each planned analysis using the Cochran-Q heterogeneity test and measured using  $\chi^2$  and  $I^2$  tests, and statistical significance was considered to be present when  $P < 0.05$ .

## RESULTS

### Description of studies included in the meta-analysis

A flow diagram of the search process is shown in Figure 1. The total search yielded 61 articles and 15 abstracts, after accessing the title and abstract, 65 studies were excluded for the following reasons: 49 did not provide data on vitamin D level, HCV status and/or SVR; 5 were basic studies; 8 were reviews, letters or editorials; 3 were duplicate studies.

Eleven studies involving 1575 individuals were included in this study<sup>[17,18,20-23,25-29]</sup>. The main characteristics of these studies are shown in Tables 1 and 2. Most of the studies were conducted in Europe and only one in North America. Eight studies evaluated vitamin D levels before and after antiviral therapy<sup>[17,18,21-23,25,28,29]</sup>, while three were interventional studies where vitamin D supplementation was conducted<sup>[20,26,27]</sup>. Most of the studies included mono-infected HCV individuals with the mean age rang-

ing from 38 to 56 years. Four studies were conducted in human immunodeficiency virus/HCV infected individuals<sup>[22,25,28,29]</sup>. With regard to vitamin D measurement, most of the studies employed radioimmunoassays ( $n = 5$ ) followed by chemiluminescence ( $n = 4$ ) and just one study employed HPLC. Basal vitamin D levels varied from 17 to 43 ng/mL in the studies selected, and most of the HCV infected individuals had genotype 1 (1068/1575) with mean viral load ranging from log 4.5-5.9 UI/mL. With regard to HCV treatment, most of the studies ( $n = 8$ ) included HCV individuals without previous treatment, where the pooled SVR rate was 46.4%.

### Vitamin D levels and sustained virological response

Different cut-off values for vitamin D were employed and in order to reduce this heterogeneity, a value of 30 ng/mL was used as the cut-off value, as most of the studies used this value to define vitamin D. Among the observational studies, a total of 1411 individuals were included. Using 30 ng/mL as cut-off value, the  $\chi^2$  test of heterogeneity was high ( $P = 0.3799$ ). There was a significant difference regarding vitamin D levels and SVR, where individuals with values higher than 30 ng/mL had a higher level of SVR. Using the random effects model by the Der Simonian and Laird method, the odds ratio was 1.57 (95%CI: 1.12-2.2) regardless of genotype (Figure 2).

A total of 1117 HCV infected individuals had low vitamin D levels (cut-off value of 30 ng/mL) representing 71% of the population studied, and most of these individuals were in the interventional studies (79.3%) as compared with the observational studies (69.9%). The highest association between vitamin D levels and SVR was observed in the study by Petta *et al.*<sup>[17]</sup> as demonstrated by the OR and CI (OR = 1.96; 95%CI: 1.02-3.79).

### Vitamin D supplementation and sustained virological response

With regard to vitamin D supplementation in HCV infected individuals in the interventional studies, the pooled estimation from 3 different studies indicated that SVR rates were higher in treated HCV individuals compared with non-treated HCV individuals. In the meta-analysis of SVR in the interventional studies where the cut-off value was 30 ng/mL, the OR was 4.59 (95%CI: 1.67-12.63) regardless of genotype (Figure 3). The test of heterogeneity (Cochran-Q = 2.86;  $df = 2$ ;  $P = 0.2395$ ), inconsistency  $I^2 = 30\%$ , and  $t = 0.2454$ . Of these studies, the OR values were higher in the study where only genotype 1 HCV individuals were included<sup>[27]</sup> (8.68) compared to the other 2 studies, one study included genotypes 1 and non-1<sup>[20]</sup> (1.90) and the other study recruited genotype 2 and 3 HCV infected individuals (5.78)<sup>[26]</sup>.

### Quality of the studies

Low heterogeneity was observed in the studies included in this meta-analysis according to the  $Q$  value for the observational (7.49) and interventional studies (2.86). The possible sources of heterogeneity across the studies were

**Table 1 Summary of the general characteristics of the included studies regarding vitamin D and hepatitis C virus (mean  $\pm$  SD)**

Study	Country	Mean age (yr)	Sample Size	Design	25(OH)D measurement	Basal mean vitamin D levels (ng/mL)
Nimer <i>et al</i> <sup>[26]</sup>	Israel	Treated: 48 $\pm$ 14 Non treated: 45 $\pm$ 10	Treated: 20 HCV infected individuals Non treated: 30 HCV infected individuals	Prospective randomized study	Radioimmunoassay (Diasorin)	Treated: 20 $\pm$ 8 Non treated: 19 $\pm$ 6
Milazzo <i>et al</i> <sup>[25]</sup>	Italy	45	93 HIV/HCV	Retrospective case-control study (clinical samples)	Radioimmunoassay (IDS)	Cases: 23.1 (15.3-35.3)
Abu-Mouch <i>et al</i> <sup>[27]</sup>	Israel	Treated: 47 $\pm$ 11 Non treated: 49 $\pm$ 7	Treated: 36 HCV infected individuals Non treated: 36 HCV infected individuals	Prospective randomized study	Radioimmunoassay (Diasorin)	Treated: 19 $\pm$ 6 Non treated: 20.5 $\pm$ 9
Bitetto <i>et al</i> <sup>[20]</sup>	Italy	Treated: 56 (42-61) Non treated: 52 (23-67)	Treated: 15 HCV infected individuals Non treated: 27 HCV infected individuals	Prospective randomized study	Chemiluminescence immunoassay (Diasorin)	NA
Soumekh <i>et al</i> <sup>[28]</sup>	United States	NA	88 HIV/HCV infected individuals	Prospective study (clinical samples)	Chemiluminescence immunoassay (Diasorin)	NA
Reiberg <i>et al</i> <sup>[29]</sup>	Austria	38	84 HIV/HCV infected individuals	Cohort (clinical sample)	NA	21.9 $\pm$ 13.8
Jazwinski <i>et al</i> <sup>[23]</sup>	United States	NA	82 HCV infected individuals	Cohort (clinical sample)	Chemiluminescence immunoassay (Diasorin)	NA
Lange <i>et al</i> <sup>[18]</sup>	Germany	45	468 HCV infected individuals	Cohort (clinical sample)	Radioimmunoassay (Diasorin)	17 (3-80)
Terrier <i>et al</i> <sup>[22]</sup>	France	39.5	189 HIV/HCV infected individuals	Cohort (clinical samples)	Radioimmunoassay (Diasorin)	18.5 $\pm$ 9.8
Bitetto <i>et al</i> <sup>[21]</sup>	Italy	47	211 HCV individuals	Cohort (clinical samples)	Chemiluminescence immunoassay (Diasorin)	20.7 (2.1-59.6)
Petta <i>et al</i> <sup>[17]</sup>	Italy	52	Cases: 196 HCV infected individuals	Transversal case-control (clinic and community sample)	HPLC	25.0 $\pm$ 9.9

NA: Not available as mean value; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HPLC: High performance/pressure liquid chromatography; 25(OH)D: 25-hydroxyvitamin D.

also explored using meta-regression analysis with the following co-variables as predictor variables: HCV genotype (1 and non-1); previous treatment (yes or no); Origin (Europe or America); method of vitamin D determination (HPLC, chemiluminescence or radiomunoassay). None of these variables interfered with the levels of vitamin D according SVR (data not shown). It is likely that this occurred because most of the studies were from Europe, including HCV genotype I individuals without previous treatment.

Although low heterogeneity was found, it was not possible to ensure high quality of all studies included in this meta-analysis. Some studies did not provide relevant data such as, mean age of the population included<sup>[23,28]</sup>, mean basal vitamin D measurement<sup>[20,23,28]</sup>, or mean HCV viral load<sup>[23,26-28]</sup>.

## DISCUSSION

Our review and meta-analysis summarize the results of eleven studies, which included a total of 1575 cases with hepatitis C, where basal 25(OH)D levels and 25(OH)D supplementation were associated with SVR in HCV patients. This updated review confirms and extends earlier results of a systematic review conducted by Cholangitis<sup>[6]</sup>, who reported that vitamin D deficiency is very frequent before liver transplantation and ranges between 51% and

92%, whereas, in the liver transplantation setting, the prevalence of vitamin D deficiency is also high.

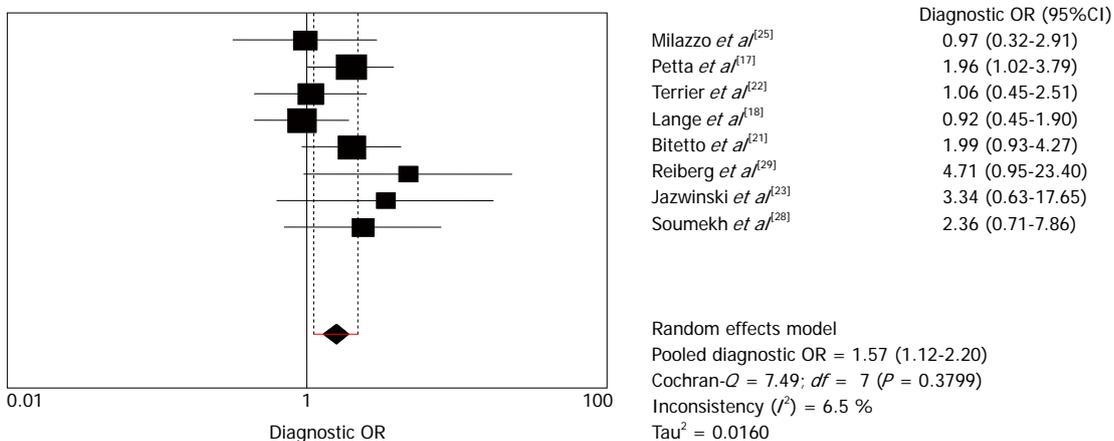
Vitamin D is metabolized by the liver and converted to 1,25-dihydroxyvitamin D<sub>3</sub>, which is the active form of the vitamin<sup>[29,30]</sup>. Individuals with chronic liver disease may have poor conversion from vitamin D<sub>3</sub> or any of its other biologically active metabolites<sup>[31]</sup>. Severe liver disease may increase the risk of vitamin D deficiency and/or there might be a relationship between vitamin D deficiency and fibrosis. Putz-Bankuti *et al*<sup>[32]</sup> and Baur *et al*<sup>[33]</sup> also showed that low levels of 25(OH)D are associated with fibrosis and suggested that low 25(OH)D levels may predict hepatic decompensation and mortality in patients with chronic liver failure. More recently, Gal-Tanamy *et al*<sup>[34]</sup> showed that vitamin D<sub>3</sub> increased the expression of the VDR and inhibited viral replication in cell culture.

Due to the observation of vitamin D deficiency in chronic liver disease patients, some studies have been conducted to evaluate vitamin D supplementation in these patients<sup>[20,26,27]</sup>. In some of these studies, it is reported that higher sunlight exposure or vitamin D supplementation should be recommended in patients with CHC<sup>[20,26,27]</sup>. In the present meta-analysis, vitamin D supplementation was related to higher SVR rates in HCV infected individuals, where the highest level was observed among genotype 1 HCV infected individuals. Although only a few studies regarding vitamin D supplementation

**Table 2 Summary of included studies regarding vitamin D and hepatitis C virus aspects**

Study	Sample size	HCV genotype	Mean viral load log <sub>10</sub> (UI/mL, average)	SVR (n)	SVR <sup>1</sup> (above/below 30 ng/mL)	Previous HCV treatment
Nimer <i>et al</i> <sup>[26]</sup>	50 HCV infected individuals	II :28 III :22	NA	Treated: 19/20 Non treated: 23/30	19/23	None
Milazzo <i>et al</i> <sup>[25]</sup>	93 HIV/HCV infected individuals	I :66 Non I :27	5.8 (5.3-6.2)	21	7/14	Naive: 31 Non responder or relapser to a previous anti-HCV therapy: 20
Abou-Mouch <i>et al</i> <sup>[27]</sup>	72 HCV infected individuals	I :72	NA	Treated: 31 Non treated: 15	31/15	None
Bitetto <i>et al</i> <sup>[20]</sup>	42 HCV infected individuals	I :32 Non I :10	Treated: 5 (3-7) Non treated: 5 (3-7)	Treated: 8/15 Non treated: 5/27	6/7	All
Soumekh <i>et al</i> <sup>[28]</sup>	88 HIV/HCV infected individuals	I or IV :77 Non I :11	NA	13	6/7	All
Reiberg <i>et al</i> <sup>[29]</sup>	84 HIV/HCV infected individuals	I :47 Non I :37	4.5 (1.4-7.6)	39	11/28	None
Jazwinski <i>et al</i> <sup>[23]</sup>	82 HCV infected individuals	I :82	NA	74	39/35	None
Lange <i>et al</i> <sup>[18]</sup>	468 HCV infected individuals	I :317 I :43 I :108	5.9 (2.3-7.7)	280	17/152	None
Terrier <i>et al</i> <sup>[22]</sup>	189 HIV/HCV infected individuals	I :84 II or III :73 IV :31 Other 1	5.9 (0-7)	61	9/52	None
Bitetto <i>et al</i> <sup>[21]</sup>	211 HCV individuals	I :95 II :63 II :38 IV-V :15	5 (2-7)	134	78/56	None
Petta <i>et al</i> <sup>[17]</sup>	196 HCV infected individuals	I :196	5 (2-8)	82	26/56	None

<sup>1</sup>According to basal vitamin D cut-off or supplementation. NA: Not available as mean value; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; SVR: Sustained virological response.

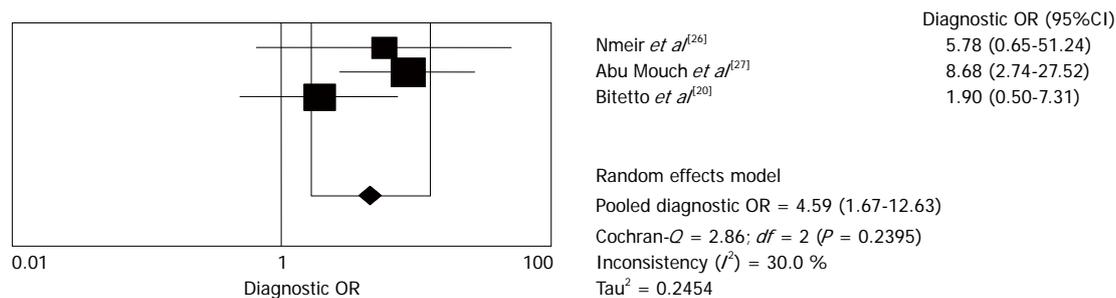


**Figure 2 Meta-analysis of 8 observational studies regarding vitamin D levels and sustained virological response against hepatitis C virus infection.**

fulfilled the eligibility criteria, different patterns were observed. The first study included only genotypes 2 and 3, the second included only genotype 1 and the third study involved genotypes 1, 2 and 3. Moreover, two of these studies were prospective and one was retrospective. Some limitations of these studies included the small number of patients, lack of vitamin D level assessment during therapy in the treatment and control groups, the design of prospective and randomized studies which were not placebo-controlled in one study<sup>[27]</sup>, and the retrospective

design of the study where immunocompromised HCV patients were supplemented with low-dose vitamin D (800 IU/d) after liver transplantation and most of the HCV patients (75%) had low vitamin D levels despite treatment<sup>[20]</sup>.

In this meta-analysis, the levels of vitamin D were also associated with SVR, although different methods of vitamin D determination were used. Lai *et al*<sup>[8]</sup> demonstrated bias and variability in 25(OH)D measurements between laboratories and between different assays [qui-



**Figure 3** Meta-analysis of 3 interventional studies regarding vitamin D levels and sustained virological response against hepatitis C virus infection.

mioluminescence and liquid chromatography-tandem mass spectrometry (LC-MS/MS)] which can significantly affect clinical decision-making. In this situation, the adoption of common standards to allow assay calibration is urgently required.

Our study is the first meta-analysis of serum 25(OH)D levels and HCV infection in observational and interventional studies. Given the very small numbers of studies available to date, additional studies, ideally from different countries and populations are needed to assess potential differences in the associations between 25(OH)D and SVR for HCV. Large differences can be observed in different populations, depending on exposure to sunlight or vitamin D supplementation, and genetic differences<sup>[23]</sup>. Moreover, patients of African and Hispanic descent are less likely to respond to standard therapy<sup>[23]</sup> probably due to polymorphisms of the interleukin (IL)-28B gene, polymorphism of the VDR or vitamin D deficiency<sup>[17,35,36]</sup>. In this meta-analysis, all the individuals were Caucasian and most lived in Europe, which could explain vitamin D deficiency in this population resulting from possible low exposure to sunlight.

Meta-analysis is an important tool for revealing trends that may not be apparent in a single study. Pooling of independent, but similar studies increases precision and therefore the confidence level of the findings. A particular strength of our study is the application of advanced statistical techniques which allowed a summary of adjusted associations across studies and over the entire range of serum 25(OH)D values, despite the very heterogeneous categorization of 25(OH)D levels in the individual studies. Our study also has important limitations. First, as data on serum 25(OH)D in individuals were not available in each study, median, midpoints and mean of the groups were used for pooling. As a result, estimates of risk may have been less accurate than if data points on each individual had been used. Second, our meta-analysis was limited by the data provided in the individual studies, and although the authors tried to obtain the raw data from the articles, not all were available. Finally, although our review searched the MEDLINE database, recent Congress of Hepatology and Gastroenterology articles, and extensive checks for completeness by cross-referencing were employed, we cannot exclude the possibility that relevant studies may have been missed.

Despite its limitations, our review and meta-analysis

support previous suggestions and provide the most comprehensive empirical evidence to date that basal serum 25(OH)D levels and vitamin D supplementation improves SVR in HCV infected individuals. However, available data are still sparse and in-depth analyses of these associations, in the context of additional longitudinal and prospective studies, are highly desirable to enable more precise estimates and a better understanding of the role of vitamin D in HCV infection.

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## COMMENTS

### Background

Hepatitis C virus (HCV) is a serious public health problem worldwide infecting more than 130 million individuals. Recently, studies have been conducted to analyze the influence of genetic and metabolic factors on antiviral response, and a recent review showed that vitamin D levels can influence HCV treatment.

### Research frontiers

Vitamin D itself is considered biologically inactive and is hydroxylated to 25-hydroxyvitamin D [25(OH)D] in the liver. Some studies have suggested that vitamin D deficiency is associated with an increased risk of cancer, cardiovascular, autoimmune and infectious diseases. However, due to the limitations of previous reviews, the authors conducted an updated systematic review and meta-analysis to comprehensively assess vitamin D deficiency with regard to antiviral therapy and the influence of vitamin D supplementation on sustained virological response.

### Innovations and breakthroughs

Previous individual studies demonstrated that high levels of vitamin D (above 30 ng/mL) or supplementation are associated to sustained virological response (SVR) in HCV infected individuals. In the present study, a meta-analysis of observational and interventional studies was conducted which proved that high levels of vitamin D (above 30 ng/mL) or supplementation are associated with SVR in HCV infected individuals.

### Applications

By showing that basal vitamin D levels or supplementation are important for high rates of SVR in HCV patients, this study may provide a future strategy for therapeutic intervention in the treatment of HCV patients.

### Terminology

HCV is an infection caused by a virus transmitted by the parenteral route. Vitamin D itself is considered biologically inactive and is hydroxylated to 25(OH)D in the liver. In the kidney, 25(OH)D is converted to 1,25(OH)<sub>2</sub>D by 1-alpha-

hydroxylase, however, it has been demonstrated that this conversion can occur in many extra-renal tissues including the liver. Finally, 25(OH)D or 1,25(OH)<sub>2</sub>D bind to the ubiquitously expressed vitamin D receptor, which regulates approximately 3% of the human genome.

### Peer review

The authors examined the influence of vitamin D levels or supplementation among HCV infected individuals. It was observed that high levels of vitamin D or supplementation are strongly associated to SVR among HCV infected individuals. The results are interesting and may represent the role of metabolic factors in HCV infection.

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## Laparoscopic cholecystectomy for a left-sided gallbladder

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### Abstract

Cholecystectomy is a common procedure. Abnormalities in the anatomy of the biliary system are common but an abnormal location of the gallbladder is much rarer. Despite frequent pre-operative imaging, the aberrant location of the gallbladder is commonly discovered at surgery. This article presents a case of a patient with the gallbladder located to the left of the falciform ligament in the absence of situs inversus totalis that presented with right upper quadrant pain. A laparoscopic cholecystectomy was performed and it was noted that the cystic duct originated from the right side. The presence of a left sided gall bladder is often associated with various biliary, portal venous and other anomalies that might lead to intra-operative injuries. The spectrum of unusual positions and anatomical gallbladder abnormalities is reviewed in order to facilitate elective and emergent cholecystectomy as well as other hepatobiliary procedures. With proper identification of the anatomy, minimally invasive approaches are still considered safe.

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**Key words:** Left sided gallbladder; Laparoscopic chole-

cystectomy; Sinistroposition of the gallbladder; Situs inversus; Bile duct anomaly; Liver anomalies; Portal vein anomaly; Liver transplant

**Core tip:** In the absence of situs inversus, left sided gallbladders are rare anomalies. They are most commonly encountered during surgery as they usually present with right sided pain and routine preoperative testing fails to identify them. Various biliary, portal venous and other anomalies are associated with left sided gallbladders and their spectrum is reviewed in this article. Recognition of these associated anomalies will help achieve safety in hepatobiliary procedures and prevent injuries.

Iskandar ME, Radzio A, Krikhely M, Leitman IM. Laparoscopic cholecystectomy for a left-sided gallbladder. *World J Gastroenterol* 2013; 19(35): 5925-5928 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5925.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5925>

### INTRODUCTION

Located to the left side of the falciform ligament, left sided gallbladders are rare anomalies and a result of a distinct embryological process. They are seldom identified pre-operatively, and as they are associated with various biliary, portal venous, and other anomalies, the surgeon must be familiar with the potential variations that he might encounter. A left sided gallbladder was encountered during a laparoscopic cholecystectomy at our institution in a patient that presented with right sided abdominal pain. Careful dissection revealed that the cystic duct was crossing from the right side and that critical view was established with the identification of the cystic artery. The spectrum of the possible anomalies associated with left sided gallbladders is wide but does not preclude the successful performance of a minimally invasive cholecystectomy or any other hepatobiliary procedure.

## CASE REPORT

The patient is a 64-year-old female with multiple medical problems including type II diabetes mellitus, a history of deep venous thrombosis and pulmonary embolism, kidney stones and hypertension who presented to the hospital with a five day history of sharp, right-sided abdominal pain radiating to her epigastric area, chest, bilateral back, right flank and right shoulder. This was the first time patient experienced this kind of pain, and she denied history of prior cholecystitis. She had a history prior extracorporeal shockwave ureteral lithotripsy, but the admitting discomfort was different from that of prior renal colic. Three months prior to the admission, the patient had colonoscopy and esophagogastroduodenoscopy, which were normal.

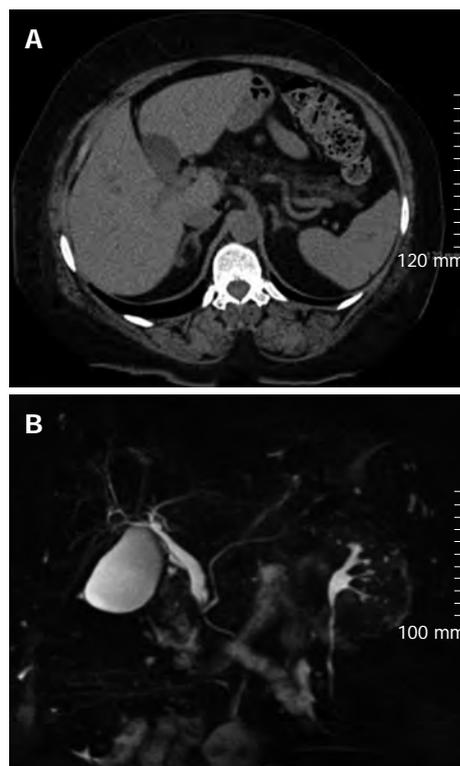
On physical examination she had stable vital signs and was afebrile. The abdomen was nondistended and nontender. Laboratory data on admission revealed a normal white cell count, normal total bilirubin, normal alkaline phosphatase, normal aspartate aminotransferase (AST) and normal alanine aminotransferase (ALT) amylase and lipase were not elevated. An abdominal ultrasound revealed gallbladder sludge with small calculi, no gallbladder wall thickening and no dilatation of the biliary tract. Computed tomography (CT) of the abdomen and pelvis showed gallstones without CT-evidence of cholecystitis, and biliary dilatation up to 9 mm. Secondary to the dilatation of the common bile duct (CBD) on the CT, magnetic resonance cholangiopancreatography (MRCP) was performed and demonstrated cholelithiasis without signs of choledocholithiasis.

During this admission, laparoscopic cholecystectomy was performed. Upon insertion of the camera into the umbilical port, the gallbladder was visualized and was located immediately to the left of the falciform ligament, and below segment III of the liver. The gallbladder wall was mildly edematous. The cystic duct and the cystic artery were identified, and it was observed that the artery was to the right of the duct. After the identification of the critical view of safety, the cystic artery and the cystic duct were clipped and divided in a standard fashion. The operation was completed without difficulties and the patient recovered and was discharged home on the second postoperative day. Pathological evaluation identified multiple small, less than 1 mm stones and the thickness of the gallbladder wall measured 3 mm, consistent with chronic cholecystitis and cholelithiasis.

The CT scan of the abdomen did not appear to demonstrate an abnormal location of the gallbladder (Figure 1A) but the finding of the gallbladder to the left of the falciform ligament was present on preoperative MRCP (Figure 1B).

## DISCUSSION

Left sided gallbladders without situs inversus are rare and have a prevalence of 0.04%-0.3%<sup>[1,2]</sup>. A distinction should be made between gallbladders that are truly left sided also



**Figure 1** Radiological images of the abdomen. A: Computed tomography scan without contrast demonstrating the gallbladder to the left of the falciform ligament; B: Magnetic resonance cholangiopancreatography showing a dilated common bile duct on coronal view.

referred to as sinistroposition and gallbladders located to the left of abnormally located right-sided round ligaments<sup>[3]</sup>. True left sided gallbladders exist because of two possible embryological etiologies. The first mechanism is due to the attachment and migration of the gallbladder to the left lobe in which case the cystic duct is in a normal anatomic position and crosses in front of the common duct from right to left, as is the case in the case reported herein<sup>[4]</sup>. The second mechanism is formation of the gallbladder by budding directly from the left side in which case the cystic duct joins the CBD or left hepatic duct from the left side<sup>[4,5]</sup>. Right-sided round ligaments on the other hand, are associated with a normal position and anatomy of the cystic duct, and with anomalous portal venous branching, which is crucial during the performance a hepatectomy, for example<sup>[3]</sup>.

Despite being truly left-sided, gallbladders with sinistroposition almost always cause right-sided symptoms when they become symptomatic making their preoperative diagnosis difficult<sup>[2]</sup>. It is believed that the visceral nerve fibers do not transpose with the gallbladder causing right-sided pain<sup>[6]</sup>. In the present case, the abnormal position of the gallbladder was only discovered at surgery, despite the patient having undergone preoperative ultrasound, CT, MRCP and endoscopic ultrasound, which is consistent with other case reports<sup>[2,3,7]</sup>. However, an intraoperative finding of a left sided gallbladder should not preclude the decision to proceed laparoscopically with minor modifications in the standard approach and port

**Table 1 Literature review of previously reported left-sided gallbladders without situs inversus undergoing surgery**

Ref.	Location	Reported number of patients	Clinical presentation	Diagnosis made pre-op?	Surgical treatment (cholecystectomy)	Comments
[1]	Hungary	1 in 2536	Right sided abdominal pain	No	Open	
[2]	The Netherlands	5 in 1764	Right sided abdominal pain	1 of 5	Laparoscopic	Sinistroposition
[3]	Japan	3 in 1621	cholecystitis, 2 incidental during liver surgery	No	Open	Emphasis on right sided round ligament/ reported 105 cases in literature until then
[5]	India	Case report	Right sided abdominal pain	No	Laparoscopic	
[6]	India	1 in 1258	Right sided abdominal pain	No	Laparoscopic	Dextrocardia present
[7]	United Kingdom	Case report	Right sided abdominal pain	No	Open	
[11]	Japan	Case report	Incidental/liver cancer	Yes	Open	Used drop infusion cholangiography for diagnosis and CT scan
[12]	Greece	Case report	Epigastric pain	No	Laparoscopic	
[13]	Ohio, United States	Case report	Right sided abdominal pain	No	Laparoscopic	Duplication of CBD
[14]	Serbia	2 patients	1 asymptomatic/1 right sided abdominal pain	No	Open	Associated with liver cysts
[15]	India	Case report	Right sided abdominal pain	No	Open	
[16]	United Kingdom	Case report	Epigastric pain	Yes	Open	Diagnosis by radio-opaque stone on the left side
[17]	Florida, United States	Case report	Right sided abdominal pain	No	Laparoscopic	Intra-op cholangiogram performed
[18]	New York, United States	Case report	Right sided abdominal pain	No	Open	Association with giardia lamblia infection
[19]	Tunis	Case report	Right sided pain	No	Laparoscopic	Normal intra-op cholangiogram
[20]	Japan	Case report	Back pain	No	Laparoscopic	Associated right portal vein anomaly
[21]	St. Louis, United States	Case report	Right sided pain	No	Laparoscopic	CBD injury because of anomalous left sided common hepatic duct
[22]	South Korea	Case report	Right sided pain	No	Laparoscopic	Pre-op percutaneous cholecystostomy with hepatic injury
[23]	Japan	Case report	Right sided pain	Yes	Laparoscopic	Preop diagnosis with DIC CT and lap CBD exploration
[24]	South Korea	3	Omphalocele with herniated liver	Yes	None	Association with omphalocele
[25]	Japan	2	Right sided pain	No	Laparoscopic	
[26]	Japan	Case report	Right sided pain	No	Open	Associated with hypoplasia of the left lobe of the liver
[27]	Japan	Case report	Right sided pain	No	Open	Right sided round ligament
[28]	Japan	Case report	Living donor transplant	No	Open	Association with portal vein anomalous branching
[29]	South Africa	Case report	Right sided pain	Yes	Laparoscopic	Diagnosed on CT pre-op
[30]	China	3	Living donor transplant	Suspected	Open	Biliary, arterial, and portal venous anomalies
[31]	Italy	Case report	Right sided pain	No	Laparoscopic	
[32]	Japan	3	Living donor transplant	Yes	Open	Portal venous anomaly

CBD: Common bile duct; DIC: Drop infusion cholangiography; MRCP: Magnetic resonance cholangiopancreatography; CT: Computed tomography.

placement. Donthi *et al*<sup>[8]</sup>, for example, placed their ports in a mirror image to a typical right-sided standard laparoscopic cholecystectomy, achieving adequate exposure and traction for dissection. Keeping in mind the possible anatomic variations associated with the condition along with careful dissection and the establishment of the critical view of safety, with or without intra-operative cystic duct cholangiography, will minimize complications. Ligation and division of the cystic duct and artery should be close to the gallbladder. The surgeon should make every effort to identify key anatomic landmarks as one would attempt to do during a standard cholecystectomy. Cases of single port cholecystectomy have even been reported in patients with situs inversus without adverse occurrences<sup>[9,10]</sup>. A more comprehensive literature review of patients with

left sided gallbladders without situs inversus undergoing surgery is summarized in Table 1.

In a conclusion, a left-sided gallbladder is an unusual anatomic variant. Patients commonly present with typical biliary colic and cholecystitis symptoms. The abnormal location might not be discovered until the start of the laparoscopic procedure. Proper anatomic identification of key landmarks will permit most or all of these procedures to be performed using minimally invasive techniques.

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## Totally laparoscopic left hepatectomy using the Torsional Ultrasonic Scalpel

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Minimally invasive surgery; Hepatectomy; Bloodless surgery; Ultrasonic Scalpel; Ultrasonic dissector; Parenchyma transection; Liver adenoma; Focal nodular hyperplasia

**Core tip:** This report describes the first total laparoscopic hemihepatectomy performed in Greece, as well as the first laparoscopic liver resection using Lotus shears. The effectiveness of the Lotus Ultrasonic Scalpel highlights the importance of surgical innovation in making minimally invasive procedures available to all surgical specialties.

Sotiropoulos GC, Stamopoulos P, Charalampoudis P, Molmenti EP, Voutsarakis A, Kouraklis G. Totally laparoscopic left hepatectomy using the torsional ultrasonic scalpel. *World J Gastroenterol* 2013; 19(35): 5929-5932 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5929.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5929>

### Abstract

Minimal invasive techniques have allowed for major surgical advances. We report our initial experience of performing total laparoscopic left hepatectomy (segments II-IV) with the Lotus (laparoscopic operation by torsional ultrasound) Ultrasonic Scalpel. The perioperative and postoperative courses of the young female patient were uneventful and she is in a good general condition without complaints 18 mo after surgery. To the best of our knowledge, this is the first total laparoscopic hemihepatectomy to be performed in Greece, as well as the first laparoscopic liver resection using Lotus shears.

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**Key words:** Laparoscopic surgery; Liver resection;

### INTRODUCTION

The development of minimally invasive hepatic resection techniques in the early 1990s established new surgical standards<sup>[1,2]</sup> and introduced highly innovative instruments such as ultrasonic dissectors, saline coagulation, and radiofrequency ablation<sup>[3-6]</sup>. We report our initial experience of performing a laparoscopic left hepatectomy with the ground-breaking Lotus (laparoscopic operation by torsional ultrasound) Ultrasonic Scalpel (S.R.A. Developments, Ashburton, Devon, United Kingdom).

### CASE REPORT

A 35-year-old asymptomatic woman with an unremarkable past medical history was referred to our department for surgical management of a liver lesion. The

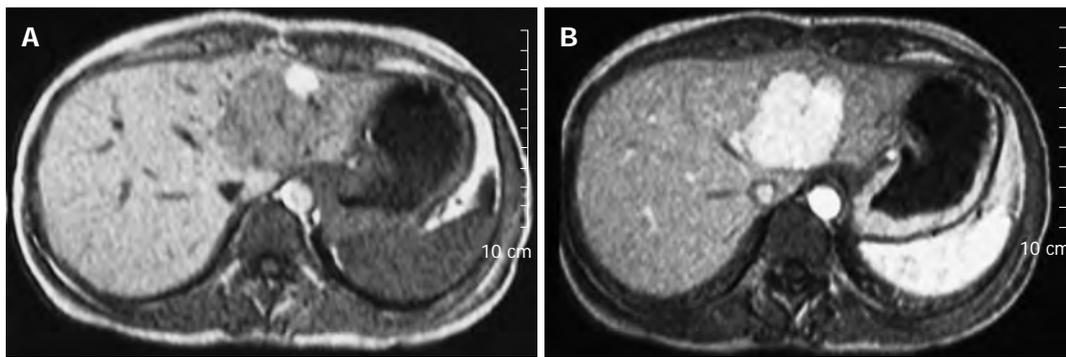


Figure 1 Magnetic resonance imaging showing the liver lesion in segments III/IV. Note the mass effect on the middle and left hepatic veins.

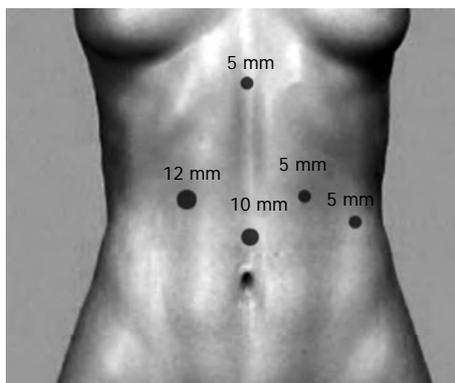


Figure 2 Patient positioning and trocar placement.

tumor had been diagnosed during work-up of elevated  $\gamma$ -glutamyltransferase (GT) (135 U/mL, normal laboratory range 7-36 U/mL) detected at premarital testing. Complete blood count, biochemical profile, liver function tests (except for  $\gamma$ GT), and tumor markers were within the normal range. There was no history of oral contraceptive use. Abdominal ultrasound showed a 5-cm isoechoic liver mass in the left hepatic lobe. Gadolinium-enhanced magnetic resonance imaging (MRI) demonstrated a 5.2-cm lesion in segments III/IV, with compression of the middle and left hepatic veins (Figure 1). A laparoscopic left hemihepatectomy was scheduled with a presumed diagnosis of liver adenoma.

### Surgical technique

With the patient in the supine position and under general anesthesia<sup>[7]</sup>, five trocar ports were placed as follows: an observation port (10 mm) 4 cm above the umbilicus; a main manipulation port (12 mm) in the midclavicular line below the right costal margin; a 5-mm port below the xiphoid process; and two 5-mm ports (for the assistant surgeon) in the left midclavicular and left anterior axillary lines, respectively (Figure 2). The operating surgeon stood between the patient's legs.

After the falciform and left triangular ligaments were transected, a replaced left hepatic artery branch was identified, clipped, and transected (Figure 3A and B). The left

branch of the portal vein was bluntly dissected (Figure 3C) and ligated with an Endopath ETS Articulating Linear Cutter (Ethicon Endo-Surgery, Blue Ash, OH, United States). The liver parenchyma was divided using the Lotus Ultrasonic Scalpel (Figure 3D and E). Non-absorbable clips were used to control the middle hepatic vein, large vessels, intrahepatic bile ducts, and the left hepatic duct. Once this had been achieved, the left hepatic vein was exposed, dissected, and divided with an Endopath ETS Articulating Linear Cutter (Ethicon Endo-Surgery) (Figure 3F). The resected specimen (segments II-IV) was removed *via* a 6-cm supraumbilical incision (Figure 4).

Total operating time was approximately 4 h. Estimated blood loss was < 400 mL. The patient had an uneventful hospital course and was discharged on post-operative day 6. Pathological evaluation of the specimen revealed focal nodular hyperplasia. The patient married 6 mo later and is currently in good health 18 mo after the procedure.

### DISCUSSION

Ultrasound-activated scalpels are safe and effective devices<sup>[8]</sup>. The Lotus Ultrasonic Scalpel introduced the concept of torsional rather than longitudinal ultrasound emissions to achieve transection and hemostasis. Its mechanism of action includes a vibratory grooved blade that generates compression forces directly into the target tissue, and a central blade that cuts as the Teflon jaw is closed. The components of the acoustic systems vibrate harmonically at 36.0 kHz. Laparoscopic torsional ultrasound shears have significant advantages over conventional cutting bipolar forceps when used to divide and coagulate pedicles in gynecological surgery. The Lotus shears are associated with shorter bisection times, less thermal damage, and more effective control of intraparenchymal blood vessels and bile ducts (a major limitation of previous devices).

To the best of our knowledge, this is the first total laparoscopic hemihepatectomy performed in Greece, as well as the first laparoscopic liver resection using Lotus shears. The effectiveness of the Lotus device further emphasizes the importance of surgical innovation in laparoscopic liver surgery.

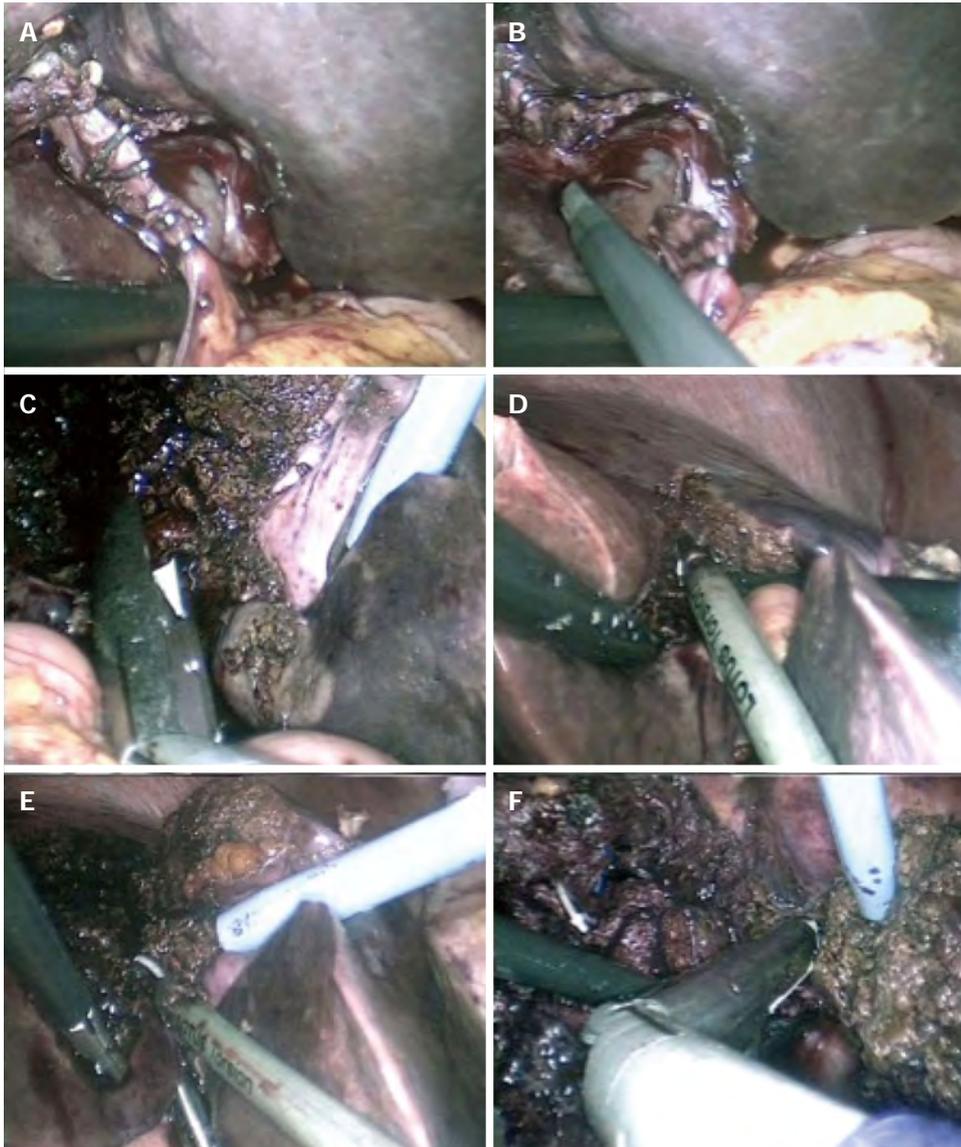


Figure 3 The operation. A and B: Identification, dissection, and clip ligation of the replaced left hepatic artery; C: Dissection of the left portal vein; D and E: Parenchymal transection using the Lotus Ultrasonic Scalpel; F: Dissection of the left hepatic vein.

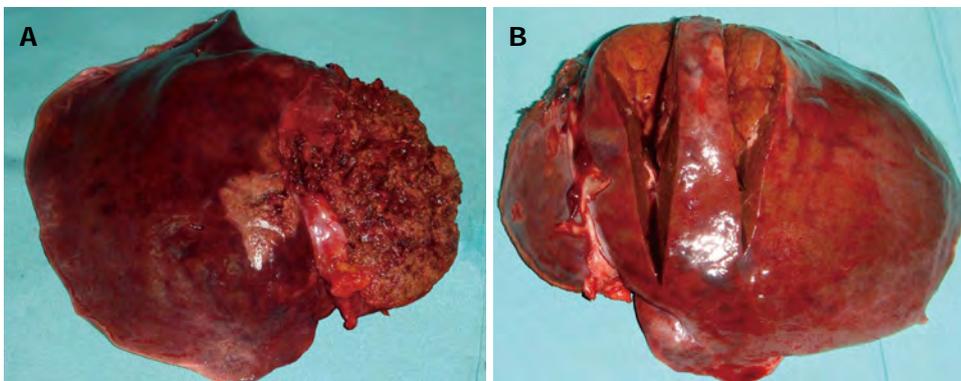


Figure 4 Left hepatectomy specimen (segments II-IV).

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## Crohn's disease and Takayasu's arteritis: An uncommon association

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### Abstract

Takayasu's arteritis (TA) and Crohn's disease (CD) are two rare autoimmune disorders; however some reports describe the presence of both diseases in the same patient. This finding has suggested the possibility that both diseases could share some common etiologic origin. We describe a case of a 13-year-old male affected by CD characterized by fever, diarrhea, weight loss, abdominal pain and elevation of inflammatory markers. Clinical and histological features from colonic specimens were consistent with CD. Treatment with steroids and azathioprine was started, however disease flared every time steroids were tapered. One year later, while still on treatment, he came back to our attention for dyspnea at rest and at night, tiredness and weakness. At physical examination a diastolic heart murmur was found as well as a left carotid artery bruit. A trans-thoracic echocardiography showed mild aortic valve insufficiency, left ventricular hypertrophy and a dilated ascending aorta with same findings at the aortic arch. A computed tomography scan showed abdominal aorta

thickening, dilated thoracic aorta and the presence of a thoracic aortic aneurysm. TA associated with CD was diagnosed and medical treatment with cyclophosphamide, steroids and aminosalicic acid was started, with good clinical response at 6 mo follow-up. We discuss the presence of possible common causes for the two diseases and the importance of differential diagnosis in those patients characterized for intractable disease.

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**Key words:** Crohn's disease; Takayasu arteritis; Intractable inflammatory bowel disease; children; Treatment

**Core tip:** It is known that both Takayasu's arteritis (TA) and Crohn's disease (CD) can present together in the same patient although this association is considered extremely rare. We would like to underline the importance of considering an alternative diagnosis in those patients characterized by intractable diseases; in our case, in fact, an intractable CD masked TA and the patient did not achieve clinical remission until he was treated with major immunosuppressive therapy; a treatment which can not be considered a standard protocol for CD.

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### INTRODUCTION

Takayasu's arteritis (TA) is a rare, chronic, relapsing large vessel vasculitis affecting the aorta and its major branches, and presenting manifestations include fatigue, weight loss, hypertension, headaches, strokes, and ischemic abdominal pain. Absence of peripheral pulses has given it the name "pulseless disease". Crohn's disease (CD)

is an idiopathic, chronic, relapsing transmural inflammation affecting primarily the gastrointestinal mucosa. The inflammatory process tends to be eccentric and segmental, often with skipped areas (normal regions of bowel between inflamed areas). Both diseases can be considered rare: in the United States, the reported incidence of pediatric CD is 4.56/100000 and the pediatric prevalence is 43/100000<sup>[1]</sup>, while the incidence of TA is almost 1 case per million. However there are some reports describing the presence of the two conditions in the same patient. It occurs usually during adulthood, while in children this association is extremely rare, but almost 1 in 10 patients with TA may develop CD or CD-like colitis.

Herein we describe the case of a child initially diagnosed as having CD who then presented with TA as well.

## CASE REPORT

A 13-year-old-boy presented with a 3 mo history of fever, diarrhea, weight loss and abdominal pain. Laboratory examination revealed elevation of inflammatory markers (erythrocyte sedimentation rate: 110 mm/h; C-reactive protein: 12.5 mg/dL) with microcytic anemia (Hb: 9.2 gr/dL; mean corpuscular volume 68 fl).

Abdominal ultrasound showed an increased terminal ileum wall thickness, while colonoscopy presented linear and aphthous ulcers with some areas of cobblestone mucosa. A biopsy showed the presence of basal plasmacytosis, an increase of lamina propria cellularity (round cells and neutrophils), basal lymphoid aggregates and epithelioid granuloma.

### *Clinical and histological features were consistent with CD*

Treatment with steroids and azathioprine was started, however disease flared every time steroids were tapered. One year later, while still on treatment, he came back to our attention for a clinical picture characterized by dyspnea at rest and at night, with extreme tiredness and weakness. At physical examination a diastolic heart murmur was found as well as a left carotid artery bruit. Transthoracic echocardiography showed mild aortic valve insufficiency, left ventricular hypertrophy and a dilated ascending aorta with same findings at the aortic arch. A computed tomography scan showed abdominal aorta thickening, a dilated thoracic aorta and the presence of a thoracic aortic aneurysm. TA associated with CD was diagnosed and medical treatment with cyclophosphamide, steroids and aminosalicic acid (ASA) was started with good clinical response at 6 mo follow-up.

## DISCUSSION

Although TA is a form of vasculitis that chiefly affects the aorta and its major branches, systemic features such as weight loss, fevers, and fatigue are found in 42%-83% of children at diagnosis of active TA<sup>[2]</sup>. At the same time musculoskeletal disease, including arthritis, arthralgia, and myalgia, is present in 12%-65% of children as well as skin manifestations, lymphadenopathy posterior reversible

encephalopathy syndrome, keratouveitis, bilateral ocular ischemic syndrome and relapsing polychondritis<sup>[2]</sup>.

TA-associated diseases also include pyoderma gangrenosum, ankylosing spondylitis, juvenile rheumatoid arthritis and inflammatory bowel disease<sup>[2]</sup>. TA in patients with CD was first described in 1970<sup>[3]</sup>, but co-existence of TA and CD has been reported in the following years<sup>[4]</sup> even if mostly in adulthood, while its presence in childhood is considered extremely rare. The expected prevalence of CD in patients with TA, if present by chance alone, is approximately 0.05%-0.2%. Thus it has been suggested that this unexpected association is more than just a coincidence<sup>[5]</sup>.

Although the pathogenesis of both diseases remains unclear some similarities have been found. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , are common in both and anti-TNF- $\alpha$  monoclonal antibody is an effective therapeutic agent for both TA and CD suggesting the presence of a common inflammatory pathway<sup>[6]</sup>. In addition, the presence of granulomatous vasculitis was found in 15 out of 25 patients affected by CD<sup>[6]</sup>; on the other hand the vasculitis of TA is characterized by granulomatous inflammation characterized by transmural inflammation of portions of the arterial wall (including the elastic laminae) and granulomas containing multinucleated histiocytic and foreign body giant cells, histiocytes, lymphocytes (which are predominantly CD4<sup>+</sup> T cells), and some plasma cells with fibroblasts<sup>[7]</sup>. Clinical details of 21 reported cases of TA associated with CD<sup>[5,8]</sup> showed that CD preceded TA in 13 reported cases as seen in the present patient. In these cases TA developed while being treated with corticosteroids, azathioprine and/or disease modifying drugs, such as 5-ASA, irrespective of the activity of CD and in one case also during infliximab treatment.

We present the interesting case of a patient affected by TA arising some months after CD. Although the exact mechanisms underlying the coexistence of the two diseases is not clear, it seems unlikely that coincidence could account for the simultaneous occurrence of these rare diseases, but the data are insufficient to allow for certainty. As explained above, it seems that a granulomatous inflammation may be considered a final method of development for many different conditions like TA and CD; however chronic granulomatous disease (CGD), Behcet disease or interleukin 17 deficiency may present with the same histological features. On this basis we could speculate that in these patients, inflammatory bowel disease could be considered an intestinal involvement of TA, rather than the coexistence of two different clinical conditions. Unfortunately there are no data on intestinal specimens in patients affected by TA and it is not clear if TA could be considered, in these cases, an extra-intestinal involvement of CD. In addition we would like to underline the importance of considering an alternative diagnosis in those patients affected by CD who do not respond to conventional treatment. In these cases, it is mandatory to rule out the presence of CGD, Behcet disease or TA.

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## Difficult polypectomy-giant hypopharyngeal polyp: Case report and literature review

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**Key words:** Esophagus; Giant fibrovascular polyp; Esophageal polyp; Fibroepithelial polyps

**Core tip:** We report an unusual case of giant hypopharyngeal polyp in a patient with anemia by chronic oozing. Giant esophageal and hypopharyngeal polyps are benign tumors rarely encountered in clinical practice; in fact, there are approximately 250 cases reported in the literature. The interesting fact is the patient regurgitated the polyp during the extraction of the echoendoscope (photo), fortunately without experiencing respiratory distress. It is rare to diagnose these polyps and it is even rarer to perform emergency surgery due to the presence of a large, regurgitated polyp that occupies most of the oral cavity.

### Abstract

Giant esophageal and hypopharyngeal polyps are benign tumors rarely encountered in clinical practice. In most cases, they are completely asymptomatic; however, despite the rarity of these tumors, interest in giant esophageal polyps derives from their degree of growth (characterized by slow growth into the esophageal lumen) and their mobility. In fact, if regurgitation occurs, they can ascend into the oral cavity and be aspirated into the airways, with potentially lethal consequences. The removal of these giant polyps is recommended. An adequate preoperative evaluation to identify the correct origin of the stalk is mandatory for a successful endoscopic or surgical treatment. A 60-year-old man was admitted to our hospital for anemia. The patient underwent gastroscopy, contrast computed tomography and endoscopic ultrasound. At the conclusion of the procedure, during the extraction of the echoendoscope, the patient began retching and regurgitated the polyp, without experiencing respiratory distress. The patient underwent a left cervicotomy and polyp dissection *via* a pharyngotomy.

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### INTRODUCTION

Giant esophageal and hypopharyngeal polyps are benign tumors rarely encountered in clinical practice; they represent approximately 0.03% of all esophageal/hypopharyngeal neoplasms<sup>[1]</sup>. The majority originate from the upper third of the esophagus, rarely from the hypopharynx, and extremely rarely from the oropharynx. They may vary greatly in size; however, they can extend along the entire length of the esophagus until reaching the gastric cavity. In most cases, they are completely asymptomatic; however, they rarely can cause anemia by chronic oozing. In addition, they can cause asphyxiation by regurgitation of the polyp into the oral cavity. The world literature contains about 110 cases of giant oropharyngeal or esopha-

geal polyps (> 5 cm); they are most often successfully treated by peroral, cervicotomic, or thoracotomy surgery; they also can be managed by endoscopy<sup>[2]</sup>.

## CASE REPORT

A 60-year-old man, with a negative medical history, was admitted to another hospital eight months ago for anemia and underwent colonoscopy (findings: negative), gastroscopy (findings: esophageal neoformation of the lower third of the esophagus), and computed tomography (CT) (findings: submucosal esophageal neoformation capturing contrast medium, extending from the cervical to the cardiac esophagus). After transfusion therapy, the patient refused any surgical treatment. He enjoyed good health until another episode of anemia occurred (hemoglobin 8.6 g/dL) and he presented at our facility. The patient underwent a gastroscopy (findings: esophageal stenosis for submucosal neoformation arising from the cervical esophagus, occupying approximately 50% of the esophageal lumen, with mucosal integrity, and protrusion of a neoformation with erosion of its apex into the gastric cavity). He also underwent a contrast CT (findings: bulky neoformation jutting into the esophageal lumen extending from the cervical to the distal esophagus with a maximum diameter of 35 mm × 46 mm, associated with increased esophageal diameter) (Figure 1). The patient then underwent an endoscopic ultrasound (EUS) (findings: thickening of the esophageal wall due to a solid, hypoechoic, neoformation, with hyperechoic areas and a vascular network, originating from the submucosa of the cervical esophagus, which affected the esophagus from cardiac portion to the upper esophageal sphincter) (Figure 2). At the conclusion of the procedure, during the extraction of the echoendoscope, the patient began retching and regurgitated the polyp, without experiencing respiratory distress (Figure 3). The patient was taken to the operating room and, under general anesthesia, a gastroscopy was repeated. It revealed that the origin of the polyp was not at the upper esophageal sphincter (UES), as previously diagnosed by CT, EUS, and the first gastroscopy; rather, it originated in the hypopharynx below the left arytenoid cartilage, with a stalk of about 3 cm in length. The patient underwent a left cervicotomy and polyp dissection *via* a pharyngotomy. The removal of the polyp was performed with a stapler (Endogia 45, Ethicon®). At the conclusion of the procedure, a dual lumen nasogastric tube was placed. On the seventh postoperative day the patient underwent a radiological evaluation with water-soluble contrast medium, which imaged good transit in the absence of extraluminal spillage. The patient began a semi-liquid diet and was discharged on the 10<sup>th</sup> postoperative day. A gastroscopic evaluation 45 d postoperatively was normal.

## DISCUSSION

Benign esophageal/hypopharyngeal tumors are rare and



Figure 1 Computed tomography (mediastinal window setting) shows bulky neoformation jutting into the esophageal lumen.

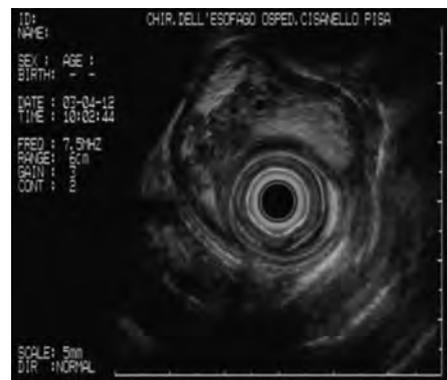


Figure 2 Endoscopic ultrasound shows a solid, hypoechoic, neoformation, with hyperechoic areas and a vascular network.



Figure 3 Giant, regurgitated hypopharyngeal polyp.

represent less than 1% of esophageal/hypopharyngeal neoplasms, *e.g.*, Moersch and Harrington<sup>[3]</sup> discovered 44 (0.59%) benign esophageal tumors in 7459 consecutive autopsies at the Mayo Clinic. Most of these tumors are intramural, represented by leiomyomas, neurofibromas, and hemangiomas; the intraluminal lesions are represented by fibrolipomas, fibromixomas, hamartomas, fibromas, and lipomas<sup>[4]</sup>. These tumors are globally ranked by the World Health Organization as fibrovascular polyps. Histologically, giant esophageal polyps are covered by

squamous epithelium with a fibrovascular axis, consisting of adipose and connective tissue to varying degrees, and a well-developed vascular network. Despite the rarity of these tumors, interest in giant esophageal polyps derives from their degree of growth (characterized by slow growth into the esophageal lumen) and their mobility. In fact, if regurgitation occurs, they can ascend into the oral cavity and be aspirated into the airways, with potentially lethal consequences. They are more frequent in males (male:female ratio = 3:1)<sup>[4]</sup>, and are usually diagnosed in the sixth or seventh decade of life; however, fibrovascular polyps have been reported in childhood<sup>[5]</sup>. About 85% are located in the upper third of the esophagus, close to the UES, and originate as small mucosal tumors, extending into the esophageal lumen due to the constant downward thrust by food ingestion and peristalsis. In the present case, the polyp was attached in an unusual site, at the level of the left anterolateral hypopharyngeal wall immediately below the ipsilateral arytenoid cartilage, with dimensions of 18 cm × 5.4 cm × 4 cm. Fibrovascular polyps usually occur as solitary lesions; however, some authors have reported synchronous polyps<sup>[6]</sup>. Due to progressive tumor growth, most patients report progressive dysphagia, initially with solid food and then with liquids. The second most frequent symptom is the regurgitation of the polyp into the hypopharynx or oral cavity with the risk of aspiration into the airways, resulting in asphyxia<sup>[7-10]</sup>. In a small percentage of patients, aspiration of the polyp may be the presenting symptom. Other symptoms include pharyngeal globus, weight loss, odynophagia, pharyngodynia, vomiting, abdominal pain, gastroesophageal reflux, hiccups, melena, and anemia<sup>[4,11]</sup>. The latter two symptoms are a result of ulceration of the apical part of the polyps due to gastric acidity or reflux of gastric contents into the esophagus. Malignant degeneration of these polyps is rare.

Fibrovascular polyps can be detected by either a barium esophagogram or gastroscopy. The former may reveal a dilated esophagus, with a gross intraluminal defect usually arising in proximity to the UES. However the examination may be entirely negative especially if the polyp remains in contact with the esophageal wall. The diagnosis, however, by gastroscopy, sometimes may be difficult or impossible because the fibrovascular polyp can completely or partially occupy the esophageal lumen, move against the esophageal wall and thus present a similar appearance to the mucosa. Diagnostic suspicion can be confirmed with a rear view, because if the terminal part of the polyp protrudes into the gastric cavity, it may image as a “clapper of a bell”; thus illustrating the circumferential space between the two walls. EUS may be useful for diagnosis because it clearly highlights the fibrovascular axis of the polyp, the echogenic aspect of adipose tissue, and the presence of anechoic areas due to its vascular network. Finally, this imaging procedure can help in a diagnosis *via* a needle aspiration. CT and magnetic resonance (MR) can be useful in confirming diagnostic suspicion and in deciding the proper surgical approach.

In particular, MR with axial coronal and sagittal scans, allows a precise identification of the stalk, an essential requirement for proper treatment. Despite a careful diagnostic process, fibrovascular polyps can be confused with intramural lesions; thus, identification of the stalk can be difficult and incorrect. In a review by Caceres *et al*<sup>[2]</sup>, 22% of barium esophagograms and 33% of gastroscopies yielded false negative results. Due to the potentially disastrous complications, removal of benign esophageal and hypopharyngeal polyps is strongly recommended. This can be achieved using a transoral, transcervical, transthoracic, or endoscopic approach, depending on the location and size of the polyp.

Two field approaches (abdominal and cervical) have also been described in patients with unusually large polyps<sup>[12]</sup>. It is clear that the correct diagnosis is essential to avoid unnecessary intervention and especially to choose the type of the intervention: surgical or endoscopic. For example, Oh *et al*<sup>[13]</sup> reported some difficulty in removing a cervical esophageal fibrovascular polyp through a right thoracotomy. Most polyps are surgically removed, especially those with a large, vascularized stalk; however, it has been performed endoscopically by dissection of the peduncle by ligature, electrocoagulation, or laser<sup>[14,15]</sup>. Generally, the removal of the polyp is curative and recurrence after resection is rare; however, some authors have reported a recurrence<sup>[16,17]</sup>.

In conclusion, giant esophageal polyps are extremely rare, benign tumors, whose removal is recommended because of the possibility of fatal consequences, bleeding and malignant transformation (rare). An adequate pre-operative evaluation to identify the correct origin of the stalk is mandatory for a successful endoscopic or surgical treatment. In addition to the removal of the giant polyp, all mucosal redundancy must be evaluated and possibly removed to avoid recurrences, which are rare but possible.

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## Obscure bleeding colonic duplication responds to proton pump inhibitor therapy

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Author contributions: Jacques J and Sautereau D reported this case; Jacques J and Loustaud-Ratti V wrote the paper; Jacques J, Legros R, Sarabi M, Carrier P and Valgueblasse V were attending doctors for the patient; Bouvier S and Fredon F performed the surgical operation; Progetti F performed pathological examinations.

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### Abstract

We report the case of a 17-year-old male admitted to our academic hospital with massive rectal bleeding. Since childhood he had reported recurrent gastrointestinal bleeding and had two exploratory laparotomies 5 and 2 years previously. An emergency abdominal computed tomography scan, gastroscopy and colonoscopy, performed after hemodynamic stabilization, were considered normal. High-dose intravenous proton pump inhibitor (PPI) therapy was initiated and bleeding stopped spontaneously. Two other massive rectal bleeds occurred 8 h after each cessation of PPI which led to a hemostatic laparotomy after negative gastroscopy and small bowel capsule endoscopy. This showed long tubular duplication of the right colon, with fresh blood in the duplicated colon. Obscure lower gastrointestinal bleeding is a difficult medical situation and potentially life-threatening. The presence of ulcerated ectopic gastric mucosa in the colonic duplication explains the partial efficacy of PPI therapy. Obscure gastrointestinal

bleeding responding to empiric anti-acid therapy should probably evoke the diagnosis of bleeding ectopic gastric mucosa such as Meckel's diverticulum or gastrointestinal duplication, and gastroenterologists should be aware of this potential medical situation.

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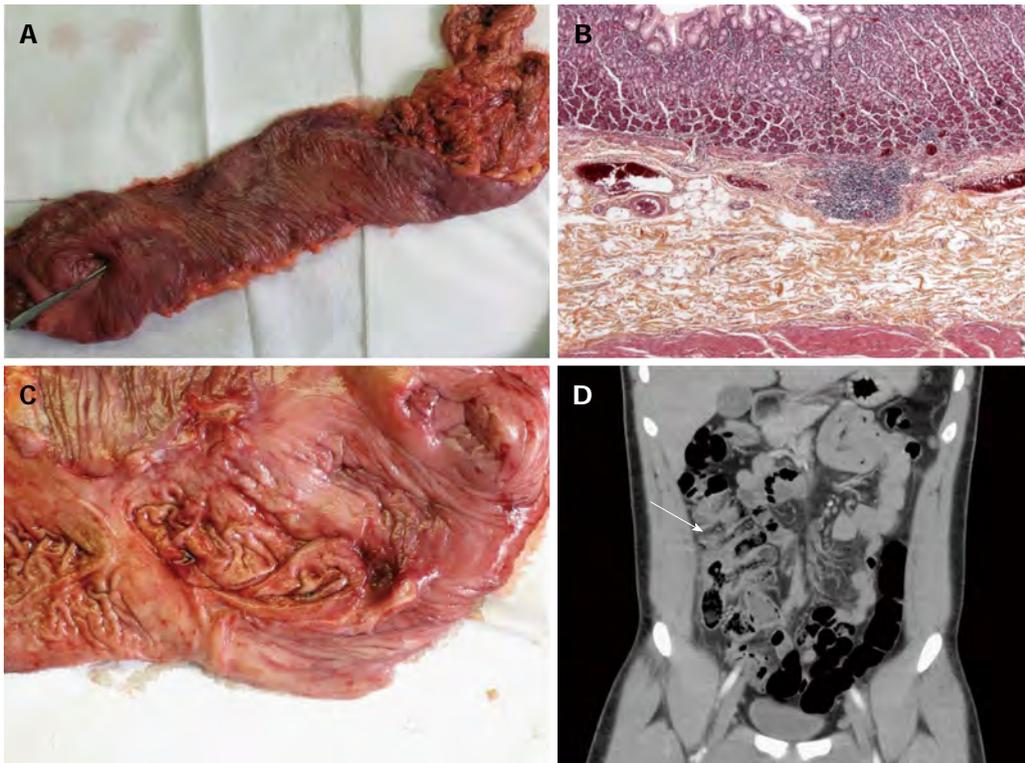
**Key words:** Colonic duplication; Gastro-intestinal duplication; Gastrointestinal bleeding; Hemostatic colorectal surgery; Proton pump inhibitor therapy

**Core tip:** Obscure lower gastrointestinal bleeding is a difficult medical situation and potentially life threatening. The collaboration between endoscopists and radiologists usually allow location of the source of bleeding, but some rare situations, such as gastrointestinal malformations, need surgical intervention to diagnose and concomitantly treat an obscure bleeding source. In terms of medical therapy, only proton pump inhibitor therapy has efficacy in peptic ulcer disease. Obscure gastrointestinal bleeding responding to empiric anti-acid therapy should suggest the diagnosis of bleeding ectopic gastric mucosa such as Meckel's diverticulum or gastrointestinal duplication, and gastroenterologists should be aware of this potential medical situation.

Jacques J, Progetti F, Legros R, Valgueblasse V, Sarabi M, Carrier P, Fredon F, Bouvier S, Loustaud-Ratti V, Sautereau D. Obscure bleeding colonic duplication responds to proton pump inhibitor therapy. *World J Gastroenterol* 2013; 19(35): 5940-5942 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5940.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5940>

### INTRODUCTION

Obscure lower gastrointestinal bleeding is a difficult medical situation which is potentially life-threatening. No



**Figure 1** Colonic duplication. A: Macroscopic analysis of the resected colonic duplication: colonic duplication with a pen showing the anastomosis with the right colon; B: Pathological analysis of the duplication: pathological analysis showing intestinal duplication with a smooth muscle coat and an ectopic gastric mucosal lining; C: Bleeding ulcer in the colonic duplication: macroscopic view of a large bleeding ulcer near the anastomosis between the duplicated and the right colon; D: Computed tomography scan coronal view of the colonic duplication: retrospective coronal reconstruction of the emergency computed tomography scan showing the duplicated right colon (arrow) which was not visible in the axial view.

medical therapy is currently available to manage these patients until endoscopic, radiologic or surgical therapy is performed after the identification of the bleeding lesion. Proton pump inhibitor (PPI) therapy is not recommended for bleeding lesions downstream of the angle of Treitz. We describe here the partial efficacy of PPI therapy in massive obscure gastrointestinal bleeding due to a bleeding ulcer in the ectopic gastric mucosa of an undiagnosed colonic duplication.

## CASE REPORT

A 17-year-old male was admitted to the emergency room of our academic hospital with massive rectal bleeding. He had reported recurrent gastrointestinal bleeding since childhood, which led to multiple hospitalizations. At 5 and 2 years before this admission he underwent exploratory laparotomies because of suspicion of a Meckel diverticulum. On admission, blood pressure was lowered to 80/55 mmHg, with a reflex tachycardia of 120 beats/min. The hemoglobin level was 5.7 g/dL. Initial resuscitation required significant volume replacement, and the transfusion of 3 blood units and 3 fresh bags of frozen plasma. High-dose intravenous PPI therapy at the dosage used in peptic ulcer bleeding (8 mg/h) was initiated.

An emergency abdominal computed tomography (CT) scan, performed after hemodynamic stabilization, was considered normal. A gastroscopy and an ileocolonosco-

py, with a water-jet device after preparation by an enema, were normal. Bleeding stopped spontaneously, and after a 24-h clinical and biological stabilization, we decided to stop the PPI therapy because of the absence of gastroduodenal ulcer disease.

Unfortunately, a further massive rectal bleed consisting of red fresh blood with melena, and hemodynamic instability occurred 8 h later. High dose intravenous PPI therapy was reintroduced and gastroscopy conducted by a French national expert in endoscopy was once again considered normal. Small bowel capsule endoscopy was then performed and his initial interpretation eliminated a bleeding lesion upstream of the angle of Treitz. PPI therapy was once again stopped and 8 h later a third massive rectal bleed occurred which led to selective mesenteric arteriography. This showed suspicious hypervascularization of the right colon. Hemostatic laparotomy was performed because of the patient's unstable condition and lack of a diagnosis. This showed long tubular duplication of the right colon, which was connected to the cecum at one end (Figure 1A), with fresh blood in the duplicated colon. Although anastomosis was not visible during the endoscopy, the capsule was seen inside the duplicated colon. Hemostatic right hemicolectomy with ileocolonic anastomosis was performed. Pathological analysis (Figure 1B) showed intestinal duplication, with intimate attachment to the mesentery, a smooth muscle coat and an ectopic gastric mucosal lining. A large ulcer with stigma of recent hem-

orrhage (Figure 1C) was present near the anastomosis. Retrospective analysis of the CT scan by a senior gastro-intestinal radiologist using coronal reconstruction agreed with our diagnosis as difficult and rare, but possible (Figure 1D, arrow). Two years after the surgery, the patient had no further gastrointestinal bleeding.

## DISCUSSION

Duplications of the gastrointestinal tract are rare congenital anomalies that can occur anywhere along its length<sup>[1,2]</sup>, and are characterized by attachment to at least one part of the tract, a layer of smooth muscle and epithelial lining resembling some part of the tract<sup>[3]</sup>. Colonic duplication is a rare abnormality, accounting for 4%-18% of all gastrointestinal duplications, and is usually located in the cecum. Tubular types are found in only 18% of cases and are most often encountered in the small and large bowel. Gastric mucosa is frequently observed in the wall of the duplications, irrespective of their site of origin in the alimentary tract<sup>[3]</sup>. Clinically, they can become a problem at any age, but the majority are discovered during the neonatal period or infancy<sup>[4,5]</sup>. They can manifest as abdominal masses<sup>[6]</sup>, bowel obstruction and gastrointestinal hemorrhage<sup>[7]</sup>. A diagnosis of intestinal duplication can be difficult and is usually performed intraoperatively.

This case highlights the diagnostic and therapeutic evaluation during massive gastrointestinal bleeding of unknown origin. First, despite all diagnostic tools available nowadays in a tertiary center, obscure gastrointestinal bleeding can sometimes be a diagnostic and therapeutic challenge. The growing efficacy of endoscopic devices (gastroscopy, colonoscopy with washing-pump, small bowel capsule endoscopy and enteroscopy) and radiologic procedures (high resolution CT scan, arteriography) tend to make the surgical procedure therapeutic only. This case highlights the unusual but still existing role of surgery in the diagnosis and treatment of massive gastrointestinal bleeds of unknown etiology<sup>[8]</sup> in challenging cases. Whereas such a role is common in cases of obscure bleeding during childhood, the usefulness of surgical exploration must not be ignored in adults. Gastrointestinal congenital malformations such as colonic duplication are a rare cause of obscure gastrointestinal bleeding. Coronal and sagittal reconstruction of CT scans can be very useful and must always be made in cases where there is an absence of diagnosis.

In terms of medical therapy, PPI therapy represents

the treatment of choice in non-portal hypertensive gastrointestinal bleeding of the upper gastrointestinal tract<sup>[9]</sup>. No drugs are approved nowadays for the management of lower gastrointestinal hemorrhage<sup>[8]</sup>. In this case, the discrepancy in the efficacy of PPI therapy and no sign of a gastroduodenal ulcer is explained by the pathology result. High dose PPI therapy probably acted on the bleeding ulcer existing in the ectopic gastric mucosa in the duplicated right colon. Rare cases have already been reported on the hemostatic efficacy of anti-acid therapy (H<sub>2</sub>-receptor antagonist or PPI) in bleeding Meckel's diverticula whose histological analysis reveals ectopic gastric mucosa in more than half of the cases<sup>[10]</sup>. Obscure gastrointestinal bleeding responding to empiric anti-acid therapy should probably suggest the diagnosis of bleeding ectopic gastric mucosa such as Meckel's diverticulum or gastrointestinal duplication.

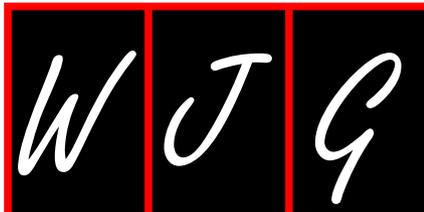
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## Capsule-odometer: A concept to improve accurate lesion localisation

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Author contributions: Karargyris A conceived and developed the capsule-odometer design, drafted the study, and performed the *in vitro* experiment; Koulaouzidis A drafted the study and performed the *ex vivo* experiment; both co-authors performed critical final draft revisions and editing.

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of-concept experiment. *In vitro* and *ex vivo* experiments with porcine small-bowel are presented herein. Further experiments have been scheduled.

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**Key words:** Capsule endoscopy; Odometer; Localisation; Hardware; Software

**Core tip:** In order to improve localisation, capsule endoscopy developers proposed the use of an applied external magnetic field. However, this affords only a rough estimate of capsule - hence lesion - localization. Therefore, a modified capsule design was proposed in 2010. It consists of a capsule fitted with protruding wheels attached to a spring-mechanism. This allows the wheels to retract or expand to fit the lumen, whilst the capsule passes through the intestine, acting as a miniature odometer. Using three-dimensional printing technology, we built a conceptual capsule prototype and miniature wheels; with the latter, we perform *in vitro* and *ex vivo* proof-of-concept experiments.

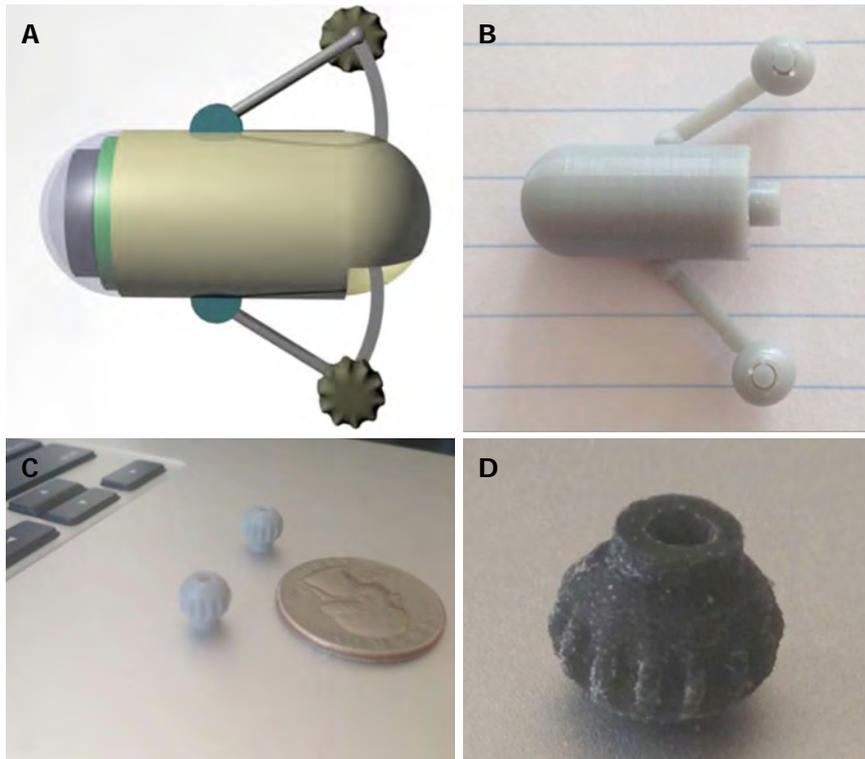
### Abstract

In order to improve lesion localisation in small-bowel capsule endoscopy, a modified capsule design has been proposed incorporating localisation and - in theory - stabilization capabilities. The proposed design consists of a capsule fitted with protruding wheels attached to a spring-mechanism. This would act as a miniature odometer, leading to more accurate lesion localization information in relation to the onset of the investigation (spring expansion *e.g.*, pyloric opening). Furthermore, this capsule could allow stabilization of the recorded video as any erratic, non-forward movement through the gut is minimised. Three-dimensional (3-D) printing technology was used to build a capsule prototype. Thereafter, miniature wheels were also 3-D printed and mounted on a spring which was attached to conventional capsule endoscopes for the purpose of this proof-

Karargyris A, Koulaouzidis A. Capsule-odometer: A concept to improve accurate lesion localisation. *World J Gastroenterol* 2013; 19(35): 5943-5946 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i35/5943.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i35.5943>

### TO THE EDITOR

Since its commercialization in the early 2000s, wireless capsule endoscopy (CE) has become a mainstream investigation for the small-bowel<sup>[1]</sup>. However, as with any technological advancement, there are performance limitations. In CE, the two main issues are low video resolution and reduced image capture rate<sup>[2]</sup>. Additionally, current systems offer somewhat crude localisation software - of



**Figure 1** Capsule odometer, conceptual design and its three-dimensional printing realisation. A: Conceptual design<sup>[10]</sup> of a capsule endoscope (herein called ODOcaps) with protruding wheels attached to a spring-mechanism; B: Three-dimensional printing technology used to build a capsule prototype; C: Wheels were initially produced with UV Curable Acrylic Plastic; D: Wheels later made of a synthetic resilient, textile-like material (TPU 92A-1).

questionable help - in mapping the imaged small-bowel. To address these issues, “smarter” CE designs have been proposed using enhanced digital circuits<sup>[2]</sup> with more efficient antennae<sup>[2-7]</sup>, and on-board video-compression<sup>[8]</sup> to conserve battery energy and increase video resolution and image capture frame rate.

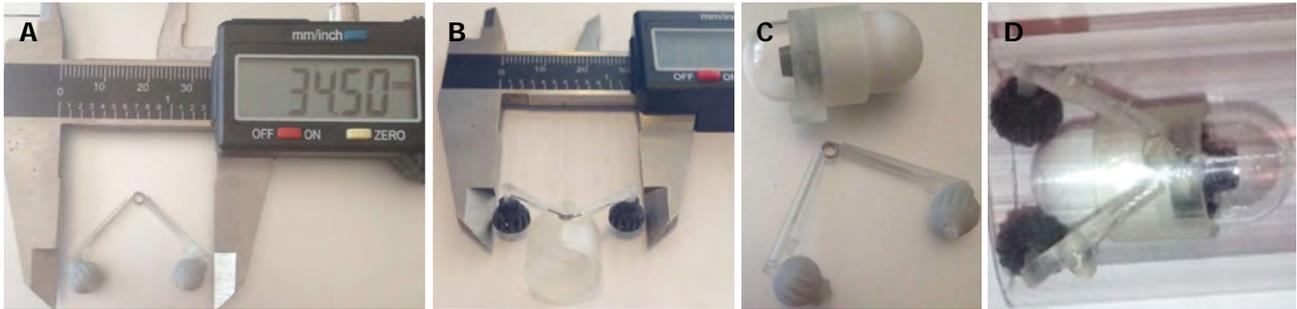
In order to improve capsule localisation, capsule endoscope developers have proposed the use of an applied external magnetic field powerful enough to manoeuvre a capsule<sup>[9]</sup>. In addition, by using geometric algorithms and applying magnetic forces, measurement of the location of the capsule as a vector in the coordinate’s space is achievable<sup>[9]</sup>. However, this method affords only a rough estimate of capsule position due to: (1) constant changes in the anatomy of the human intestine; and (2) all measurements are taken relative to the externally applied magnetic field.

In 2010<sup>[10]</sup>, we proposed a modified capsule design incorporating localisation and stabilization capabilities. The proposed design consists of a capsule fitted with protruding wheels attached to a spring-mechanism (Figure 1A). These springs allow the wheels to retract or expand to fit the lumen whilst the capsule passes through the intestine. This would then lead to more accurate location information, hence accurate lesion localisation, in relation to the onset (pyloric opening) of the investigation. Furthermore, this capsule could theoretically allow stabilization of the recorded video as any erratic, non-forward movement through the gut is minimised.

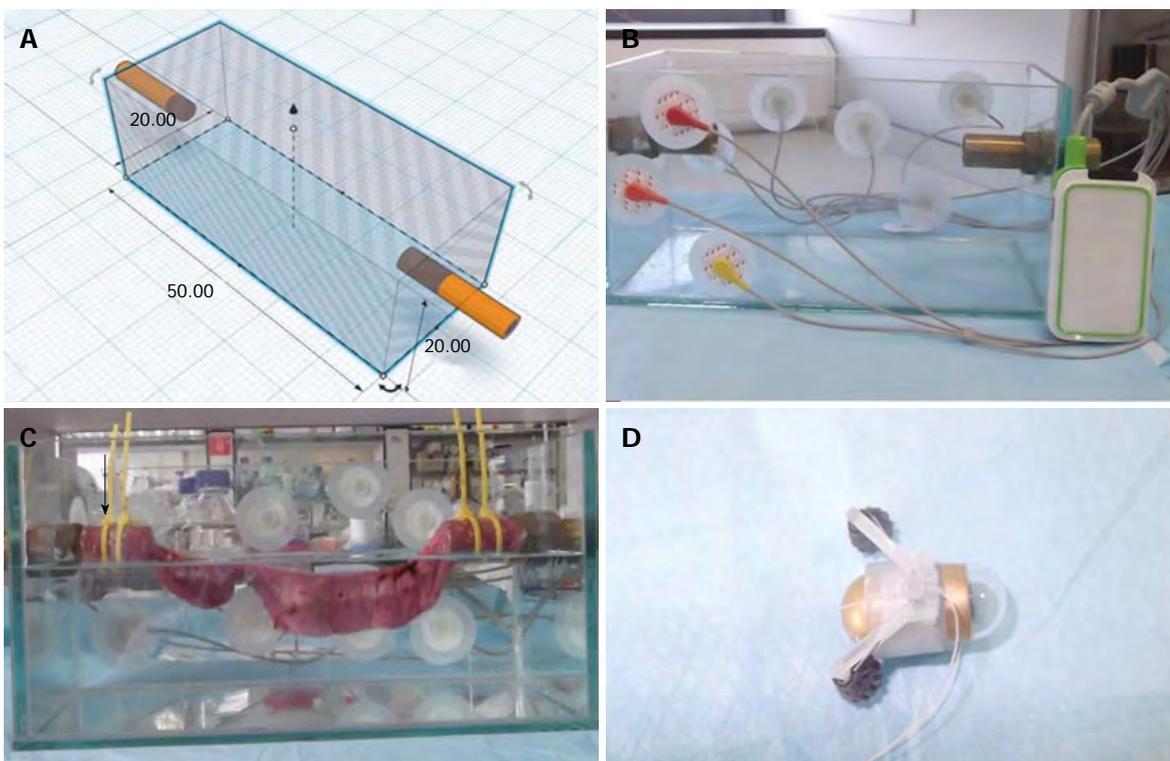
Therefore, we aim to test the feasibility of the pro-

posed design *in vitro* and *ex vivo*. Three-dimensional (3-D) printing technology<sup>[11,12]</sup> was used to build a conceptual capsule prototype (herein called ODOcaps) (Figure 1B). Furthermore, miniature wheels were initially produced with UV Curable Acrylic Plastic (Figure 1C). They were later re-designed and made of a synthetic resilient, textile-like material (TPU 92A-1) (Figure 1D). This material is produced *via* laser sintering, a process similar to stereo-lithography that uses temperature-sensitive powder (instead of UV-sensitive liquid), causing the powder to become a coherent mass without melting<sup>[13]</sup>. For the tread area of the wheels, two designs were considered: smooth or tractor-tread and tested on various types of surfaces. The tractor-tread design of the wheels was selected because it could - theoretically - achieve better traction and full rotation of the wheels when in contact with the intestinal mucosa.

Thereafter, the wheels were inserted in 3-D printed, L-shaped miniature tubes (UV Curable Acrylic Plastic; Figure 2A) allowing almost frictionless rotation. The tubes along with the wheels were attached to a spring (stainless steel torsion spring<sup>[14]</sup> with 90° deflection and 0.093 inch pounds torque from Associated Spring Raymond) to allow extension/retraction of the wheels, Figure 2B. Thereafter, spring with wheels was clipped onto a 3-D printed ring (11.5 mm in diameter made of UV Curable Acrylic Plastic; Figure 2B and C). The ring has two (1 mm) holes, diametrically opposite to each other. The ring was designed to tightly fit on conventional CE



**Figure 2** *In vitro* experiment. A: Wheels inserted in three-dimensional printed, L-shaped miniature tubes. Tubes along with the wheels attached to a spring; B: stainless steel torsion spring with 90° deflection and 0.093 inch pounds torque from Associated Spring Raymond allows extension/retraction of the wheels; C: Three-dimensional printed ring (11.5 mm in diameter made of UV Curable Acrylic Plastic on a demo PillCam® SB2); D: Inserted into one end of a translucent tube and pulled through by a silk string.



**Figure 3** *In vivo* experiment. A and B: Glass tank (50 cm × 20 cm × 20 cm) with fix points for the intestine (metal tubes) and entry points for the capsule device, C: Standard simulated intestinal environment created by mounting 32 cm long, freshly harvested porcine small-intestine to both ends of a fluid-filled tank (arrow); D: Capsule device (assembled ring with wheels on spring on a MiroCam®) was inserted into the suspended bowel *via* one of the metal tubes.

systems (PillCam® SB2, Given® Imaging Ltd., Israel; MiroCam®, IntroMedic Ltd., South Korea) and the holes were used for the insertion of a 0.5 mm silk string.

For the *in vitro* experiment, a translucent polycarbonate tube (internal diameter 22 mm and 50 cm long; Figure 2D) was used. The tube characteristics mimic the diameter and slippery surface of an adult small-bowel. The assembled ring with wheels on spring was fitted on a demo PillCam® SB2 and was inserted into one end of the tube; thereafter, it was pulled through by the string (Figure 2D). While the capsule was pulled along the tube, the wheels were observed rotating constantly without any skidding effect. A video that demonstrates this experiment is available at: <http://dl.dropbox.com/u/7591304/Capsule.mov>.

For the *ex vivo* experiment; a glass tank (50 cm × 20 cm × 20 cm) with fix points for the intestine (metal tubes) and entry points for the assembled device was constructed (Figure 3A and B)<sup>[15]</sup>. A standard simulated intestinal environment (Figure 3C) was created by mounting a 32 cm long, freshly harvested porcine (Large White x Landrace 15-mo-old female sow) small-intestine to both ends of a fluid-filled tank<sup>[15]</sup>. The tank was filled with Normal Tyrode's Solution - Base1 (NTS-1) and Base2 (NTS-2), (Dr. Lohmann Diaclean GmbH, Germany), diluted with 9l of sterile water. Thereafter, the assembled ring with wheels on spring fitted on a MiroCam®) was inserted into the suspended bowel *via* one of the metal tubes (Figure 3D). Consistency of wheels' rotation was

validated by utilising an all-purpose endoscope, Findoo MircoCam (dnt® GmbH, Germany). The latter was introduced from the same end as the capsule and recorded the wheels movement while following the moving capsule. A video is available at: <http://dl.dropboxusercontent.com/u/7591304/1035301R.AVI>

In conclusion, *in vitro* and *ex vivo*, “proof of concept” experimentation based on a conceptual CE design (Figure 1A and B) - that at least in theory offers enhanced localisation capabilities - showed promising preliminary results. Further elaborate experiments (*i.e.*, at first stage, force measurements and construction of a functional prototype)<sup>[16]</sup> and *in vivo* experiments with this prototype are essential and currently under way.

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## Medical influences, surgical outcomes: Role of common medications on the risk of perforation from untreated diverticular disease

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important in the old and frail patient where an eventual surgical treatment may not always be possible.

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### Abstract

Numerous drugs, largely used in the wards or at home, have a significant influence on patients with untreated diverticular disease. The consequences can be disastrous, may require an emergency operation, postoperative intensive care, and overall influence the patient's length of stay and the final outcomes. Bearing these considerations in mind the routine or chronic administration of pain-killers, steroids and non-steroidal anti-inflammatory should be balanced in patients with known diverticular disease as it normally happens with other conditions potentially affected by these drugs (*i.e.*, peptic ulcer disease or chronic obstructive pulmonary disease). This is even more important in the old and frail patient where an eventual surgical treatment may not always be possible.

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**Key words:** Perforation; Diverticular disease; Medications; Drugs; Risk factor

**Core tip:** Numerous drugs have an influence on patients with untreated diverticular disease. This is even more

### INTRODUCTION

Colonic diverticular disease is a common disease with a prevalence that increases with aging (65% by 80 years)<sup>[1]</sup>. A minority of patients (15%) experience severe complications. Pseudodiverticula are the most common and usually composed of the mucosal and submucosal layers. Therefore, they act as locus minoris resistentiae on the bowel wall and increase the predisposition towards perforation. Abscess formation, purulent or fecal peritonitis are the most common consequences of perforation and are associated with a high morbidity, intensive care requirements, prolonged hospital admissions and increased mortality (12%-36%)<sup>[1,2]</sup>. Conditions that predispose to an increased intraluminal pressure or reduced resistance of the diverticular mucosa can lead to perforation<sup>[1]</sup>. In this view, excessive colonic segmentation may increase intracolonic pressures and the stress forces acting on the diverticular mucosa<sup>[3]</sup>, while impairment of the mucosal barrier of the diverticulum may lead to mucosal weakening through modifications of the secretion of protective mucus<sup>[3]</sup>. Numerous drugs have such effects on patients and therefore increase the risk of perforation from colonic diverticula. The association of perforated diverticular disease with these drugs has been described in various studies. The diverticular disease is usually untreated at the

time of perforation and sometimes even undiagnosed as some patients are unaware of its presence until the perforation manifests.

## NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used classes of medications. On large surveys 17% of the general population assumes NSAIDs as chronic anti-inflammatory medications or for long-term pain control, and prescriptions for generic ibuprofen, naproxen and selective inhibitors of the cyclooxygenase 2 enzyme exceed every year the cost of billions<sup>[4]</sup>. A description of the pathophysiological and clinical effects of NSAIDs on the normal colonic mucosa is essential for a proper understanding of their influence on segments affected by diverticular disease. NSAIDs manifest their harmful action on the mucosa through the inhibition of the COX enzymes<sup>[5,6]</sup>. Cyclooxygenase (COX)-1 normally synthesizes protective prostaglandins while COX-2 is pro-inflammatory. In the first case, the lack of protective prostaglandins weakens the diverticular mucosa to noxious agents, in the second the inflammatory reaction in cases of microperforation of the diverticula is diminished and therefore the ability to contain the extracolonic contamination<sup>[5,6]</sup>.

NSAIDs have long been associated with complications in the upper gastrointestinal tract. More recently, adverse effects upon the small and large intestine have become more recognized and reported. Individuals who regularly take NSAIDs have a significantly higher incidence of lower intestinal lesions when compared with non-NSAID takers, and such risk increases with the duration of NSAIDs ingestion. NSAIDs have been associated with a particular form of colitis<sup>[7]</sup> that present with diarrhoea, anemia and non specific abdominal pain<sup>[8]</sup>. Endoscopy revealed flat ulcers in the entire colon similar to ulcerations and erosions found in the small bowel<sup>[8,9]</sup>. The median time from onset of symptoms to diagnosis was 1.8 years (range, 0.0-11.5 years)<sup>[8]</sup> and prolonged use of NSAIDs increased the risk of mucosal damage more than the short use<sup>[7]</sup>. In portions of large bowel not affected by underlying diseases numerous cases of strictures were also reported<sup>[10-15]</sup>. Such lesions appeared on endoscopy as concentric “diaphragm-like” strictures similarly to those described in the small bowel<sup>[8,9]</sup>. Finally, NSAIDs-induced perforations have been described especially in the cecum<sup>[16-19]</sup>, usually caused by more distal strictures<sup>[10,11]</sup>.

NSAIDs are frequently used to treat concomitant arthritic or cardiovascular diseases and not necessarily prescribed to alleviate symptoms in patients with diverticular disease<sup>[20]</sup>. Most patients are even unaware of the presence of diverticular disease until the perforation occurred<sup>[20]</sup>. In segments of the colon affected by diverticular disease NSAIDs increased the risk of bleeding<sup>[21-24]</sup> and perforation of diverticula. Six retrospective case-

controls studies have analysed the association between perforated diverticular disease and NSAIDs, making it the most studied class of drugs with regards to such association (Table 1)<sup>[20,25-29]</sup>. The incidence of NSAIDs in patients with perforated diverticular disease was compared with healthy controls<sup>[20,25,27]</sup> or with patients having simple non-perforated diverticular disease<sup>[20,26,28]</sup>. Overall, NSAIDs were present in 10% of patients with perforated diverticular disease (118/1182) and 3.8% of controls (391/10385), sub-classified in 3.4% in healthy people (341/9950) and 11.5% in non-perforated diverticular disease (50/435). When compared with healthy controls, OR for the use of NSAIDs in patients with perforations was 1.5 (95%CI: 1.01-2.3) for Humes *et al*<sup>[27]</sup>, 1.8 (95%CI: 0.96-3.4) for Mpofo *et al*<sup>[25]</sup>, 2.1 (95%CI: 1.3-3.4) for Goh *et al*<sup>[20]</sup>. When compared to simple diverticular disease OR were higher: 3.6 (95%CI: 1.5-8.4) for Piekarek *et al*<sup>[28]</sup>, 4.6 (95%CI: 1.7-12.5) for Goh *et al*<sup>[20]</sup>, 4.8 (95%CI: 1.6-14.8) for Corder<sup>[26]</sup>. ORs results are consistent among studies and indicate a higher presence of NSAIDs in patients experiencing perforation compared to both control groups. Interesting, higher ORs are present when perforated patients are compared to non-perforated ones as if the presence of diverticula increase the predisposition to perforation from NSAIDs compared to healthy subjects.

## CORTICOSTEROIDS

Corticosteroids are potent anti-inflammatory and immunosuppressive drugs used for a number of common and rare diseases. It is estimated that up to 0.9% of the general population receives oral administrations of corticosteroids, and 22% of these patients continue the treatment for longer than 6 mo. The most frequent indications are respiratory diseases (80%) followed by pathologies of the musculoskeletal system (12%) and the skin (10%)<sup>[30]</sup>. Arthropathies are most likely to require a chronic treatment compared to the other indications<sup>[30]</sup>.

The relationship between chronic rheumatic diseases, long-term use of corticosteroids and complications from diverticular disease has long been investigated from different perspectives. In a large study on patients with rheumatoid arthritis corticosteroids were associated with perforations of the lower gastrointestinal tract and death<sup>[31]</sup>. At the same time, patients with chronic rheumatic conditions experienced a six-fold increase of death from complicated diverticular disease than the general population<sup>[32]</sup>. The long-term use of corticosteroids has been described in chronic rheumatic patients suffering from perforated diverticular disease<sup>[33-37]</sup>. Sporadic cases of diverticular perforation have also been reported in patients following neurosurgery operations<sup>[38]</sup>, transplants<sup>[39-43]</sup> or under steroidal treatment for asthma and cancer<sup>[44]</sup>. In some patients the diverticular disease was even unsuspected until the fatal event happened<sup>[34,38]</sup>.

Corticosteroids may increase the likelihood of clinically-manifested diverticular perforations by both etiologic and contributing mechanisms. Steroidal use inhibits

**Table 1** Case-controls studies investigating the association of non-steroidal anti-inflammatory drugs, steroids, opioids, calcium channel blockers and perforated diverticular disease

Author	Country	Drugs	Patients (n)	Control group (n)	OR
Goh <i>et al</i> <sup>[20]</sup>	United Kingdom	NSAIDs	20	HC (600), DD (125)	2.1 (95%CI: 1.3-3.4) for HC 4.6 (95%CI: 1.7-12.5) for DD
Mpofu <i>et al</i> <sup>[25]</sup>	United Kingdom	NSAIDs	64	HC (320)	1.8 (95%CI: 0.96-3.4)
Corder <i>et al</i> <sup>[26]</sup>	United Kingdom	NSAIDs	-	DD	4.8 (95%CI: 1.6-14.8)
Humes <i>et al</i> <sup>[27]</sup>	United Kingdom	NSAIDs	899	HC (8980)	1.5 (95%CI: 1.01-2.3)
Piekarek <i>et al</i> <sup>[28]</sup>	Sweden	NSAIDs	54	DD (183)	3.6 (95%CI: 1.5-8.4)
Mpofu <i>et al</i> <sup>[25]</sup>	United Kingdom	Steroids	64	HC (320)	31.9 (95%CI: 6.4-159.2)
Corder <i>et al</i> <sup>[26]</sup>	United Kingdom	Steroids	-	DD	13.2 (95%CI: 1.81-96.5)
Humes <i>et al</i> <sup>[27]</sup>	United Kingdom	Steroids	899	HC (8980)	2.7 (95%CI: 1.6-4.6)
Piekarek <i>et al</i> <sup>[28]</sup>	Sweden	Steroids	54	DD (183)	28.3 (95%CI: 4.8-165.7)
Humes <i>et al</i> <sup>[27]</sup>	United Kingdom	Opioids	899	HC (8980)	2.2 (95%CI: 1.6-3.0)
Piekarek <i>et al</i> <sup>[28]</sup>	Sweden	Opioids	54	DD (183)	4.5 (95%CI: 1.7-12.2)
Morris <i>et al</i> <sup>[31]</sup>	United Kingdom	Ca <sup>2+</sup>	120	HC (480)	0.4 (95%CI: 0.2-0.9)
Humes <i>et al</i> <sup>[27]</sup>	United Kingdom	Ca <sup>2+</sup>	899	HC (8980)	0.54 (95%CI: 0.24-1.24)
Piekarek <i>et al</i> <sup>[28]</sup>	Sweden	Ca <sup>2+</sup>	54	DD (183)	0.14 (95%CI: 0.02-0.95)

NSAIDs: Non-steroidal anti-inflammatory drugs; Ca<sup>2+</sup>: Calcium-channels blockers; HC: Healthy control; DD: Non-perforated diverticular disease.

the cyclo-oxygenase enzyme in the gut that normally produces prostaglandins with local protecting effects<sup>[1]</sup>. Prostaglandins enhance the gut mucosal barrier by stimulating the secretion of mucin and bicarbonate and increasing the local blood flow<sup>[45]</sup>. Their absence predisposes the mucosa to the effects of noxious agents such as bacteria and toxins<sup>[45]</sup>. Additionally, corticosteroids are potent immunosuppressant that masks the immune response to local inflammations and small perforations. The ability of the body to contain small perforations is therefore impaired, their local effects are therefore increased and even the classic clinical symptoms may be masked until advanced contaminations eventually become evident<sup>[46]</sup>.

Four case-controls studies have investigated so far the association of perforated diverticular disease and corticosteroids (Table 1)<sup>[25-28]</sup>. In two studies controls were healthy people<sup>[25,27]</sup> and in the rest non-perforated diverticular disease<sup>[26,28]</sup>. Overall, corticosteroids were present in 4.4% of patients with perforated diverticular disease (49/1112) and 0.7% of controls (71/9560), sub-classified in 0.7% in healthy people (68/9300) and 1.2% in non-perforated diverticular disease (3/260). All studies confirmed an increased risk of perforation with their use that was 2.7 (OR = 1.604.6) according to Humes *et al*<sup>[27]</sup>, 13.2 (RR, 95%CI: 1.81-96.5) for Corder<sup>[26]</sup>, 28.3 (OR, 4.8-165.7) for Piekarek *et al*<sup>[28]</sup>, and 31.9 (OR, 95%CI: 6.4-159.2) for Mpofu *et al*<sup>[25]</sup>. However, a more careful analysis shows that the risk increase presented by Humes *et al*<sup>[27]</sup> is markedly lower than those reported by the other authors<sup>[25,26,28]</sup>. No direct comparison of the corticosteroids incidences is possible for the control groups because of the above-mentioned heterogeneity among studies (two analyse healthy people and two non-perforated diverticular diseases). Still, case groups present similar patients with perforated diverticular disease in all four studies and therefore incidences can be directly compared. In the study of Humes *et al*<sup>[27]</sup> only 2.2% (20/899) of perforated patients use corticosteroids compared to 10%-17%

reported by the others (10/64 = 16%, 10/95 = 10.5%, and 9/54 = 17%)<sup>[25-28]</sup>. This could explain the lower increase in the risk conferred by corticosteroids reported by Humes *et al*<sup>[27]</sup> compared to the others. The main difference noted among these studies is that the report of Humes is based on a nationwide database prospectively-maintained by local general practitioners (General Practice Research Database, United Kingdom). This database has provided a larger number of patients than any other local-based study, possibly giving more reliable epidemiological figures ( $n = 8980$  controls,  $n = 890$  cases).

## OPIOIDS

Opioids are common analgesics used to control pain in acute (*i.e.*, postoperative pain) and chronic conditions (*e.g.*, oncological pain, arthritis and headaches). Although they are not the first choice according to the World Health Organization analgesic ladder, it is still estimated that up to 90% of the population use them at least once in the lifetime while 0.56% are chronic users (greater than 6 mo assumption), especially elderly women<sup>[47]</sup>. The overall gastrointestinal effects consist in depression of the peristalsis with clinical manifestations of nausea, vomiting and constipation<sup>[48]</sup>. Opioids act on gut motility by decreasing the autonomic activity of the central nervous system and by binding to the mu- and kappa-receptors of the myenteric and submucosal plexuses in the gut<sup>[49,50]</sup>. The pathophysiological effects consist in an increase in the frequency of non-propulsive phasic contractions of the colon and decreased to absent propulsive migrating contractions<sup>[51]</sup>. The increase of non-propulsive contractions accounts for the higher intraluminal pressures. In fact, the administration of morphine produces high intraluminal sigmoid pressures in segments with colonic diverticula through the production of peristaltic segmentations<sup>[52-54]</sup>. These higher pressures may contribute either to the production of new diverticula or, in an already diseased segment, to

the perforation of some of them. On the other side the absence of propulsive complexes is responsible for the constipation and increased frequency of ileus. The reduction in the transit time may therefore prolong the exposure of the diverticular wall to potential pathogens<sup>[1]</sup>.

Due to all these premises, the safety of administering opioids in patients with diverticular disease was questioned early in the medical literature<sup>[52]</sup>. However, the association between opioids and perforated diverticular disease is one of the least examined compared to the other classes of drugs. The first study that report the frequency of opioids use in patients with perforation from diverticular disease is based on a large cross-sectional study in the Norwich area (United Kingdom) that found opioids were used by 26% of the population presenting with diverticular perforation<sup>[2]</sup>. More recently, both case reports<sup>[55]</sup> and case-control studies<sup>[27,28]</sup> have further reported on the association among opioids and perforation from diverticular disease. In case-controls studies data were collectively presented for drugs used as required or regularly with no differentiation according to the duration or regularity of the assumption (Table 1)<sup>[27,28]</sup>. In the study of Piekarek *et al.*<sup>[28]</sup>, patients with diverticular disease were retrospectively examined and divided in two groups according to the perforation status. In the perforated group (case group) the use of opioids was present in 20.4% of patients ( $n = 11/54$ ) while in the non-perforated group (control group) it was 6.0% (11/183). This corresponded to an OR for perforation with opioids of 4.5 (95%CI: 1.7-12.2). Differently from Piekarek *et al.*<sup>[28]</sup>, Humes *et al.*<sup>[27]</sup> conducted a larger population-based study gathering data from the General Practice Research Database. In their study controls were healthy people not affected by the diverticular disease. Current opioids use was present in 6.3% (57/899) of perforated cases (case group) *vs* 2.4% (218/8980) of healthy patients (control group) with a lower OR than that reported by Piekarek *et al.*<sup>[28]</sup> (2.2, 95%CI: 1.6-3.0)<sup>[27]</sup>. Both studies confirm that the current use of opioids increases the possibility of diverticular perforation. The different ORs observed can have different explanations. First, it is possible that differences not reported in the regularity or duration of opioids have significant influences on the occurrence of perforation. Second, the different control groups (healthy controls *vs* non-perforated diverticular disease) may provide a different baseline level for the calculation of the added risk towards perforation derived from the use of opioids, similar for the data reported on NSAIDs.

## CALCIUM CHANNEL BLOCKERS

Calcium channel blockers are a common class of drugs frequently prescribed in elderly people to treat hypertension and ischemic heart disease. They act by blocking the calcium channels in smooth muscle cells and therefore relaxing the contraction of non-voluntary musculature. Although this effect is desirable on peripheral circulation, to an extent it influences also the gastrointestinal motility

and has been used to treat pathologic contractions of the gastrointestinal tract (*i.e.*, anal fissures from increased anal sphincter tone). The muscle-relaxant properties of these medications could have a beneficial effects in reducing intracolonic luminal pressures<sup>[56]</sup>. At the same time, some of them also increase the mucosal vascular flow therefore acting on the second main risk factor for diverticular perforation (weakened mucosal barrier)<sup>[57,58]</sup>.

So far, three case control studies have analyzed the effects of Calcium channel blockers on the likelihood of perforation from diverticular disease (Table 1)<sup>[3,27,28]</sup>. In all studies the use of such medications was more frequent in controls than in patients that experienced perforated diverticular disease: 15% (72/150) in healthy controls *vs* 6.7% (8/120) in perforated patients for Morris *et al.*<sup>[3]</sup>, 1.2% (104/8980) in healthy controls *vs* 0.7% (6/899) in perforated patients for Humes *et al.*<sup>[27]</sup>, and 11.5% (21/183) in patients with simple diverticular disease *vs* 3.7% (2/54) in perforated patients according to<sup>[28]</sup>. These data corresponded to ORs of 0.4 (95%CI: 0.2-0.9)<sup>[3]</sup>, 0.54 (95%CI: 0.24-1.24)<sup>[27]</sup>, 0.14 (95%CI: 0.02-0.95)<sup>[28]</sup>. Among the three reports the only one in which the association was not statistically significant was the one of Humes *et al.*<sup>[27]</sup> although the authors still suggested “a potentially protective role”. The differences among this study (large population-based) and the others have already been outlined.

## OTHER DRUGS

Few other classes of drugs have been sporadically investigated. Antimuscarinic drugs are commonly prescribed for depression, psychoses, but also as muscle relaxants for overactive bladder. Their characteristics could also influence the gastrointestinal musculature and prevent excessive contractions and therefore perforations from diverticular disease. However, the only study that compared healthy controls *vs* patients with perforated diverticular disease failed to provide a significant association<sup>[3]</sup>. Statins also were investigated in one study<sup>[27]</sup> for their potential anti-inflammatory qualities that could protect the diverticular mucosa<sup>[59]</sup>. Current use of a statin was associated with a lower risk of perforation (OR = 0.44, 95%CI: 0.20-0.95)<sup>[27]</sup>.

## CONCLUSION

Numerous drugs, largely used in the wards or at home, have an influence on patients with untreated diverticular disease. The consequences elicited can be disastrous, would ideally require an emergency operation with post-operative intensive care monitoring for definitive treatment, and influence the overall length of stay and final outcomes. Bearing these considerations in mind, the routine or chronic administration of pain-killers, steroids and non-steroidal anti-inflammatory should be balanced in patients with known diverticular disease as it normally happens with other associated conditions that could be affected by these drugs (*i.e.*, peptic ulcer disease or chron-

ic obstructive pulmonary disease). This is even more important in old and frail patients in which an eventual surgical treatment may not always be a possibility.

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## Liver transplantation in alcoholic liver disease current status and controversies

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### Abstract

Alcoholic cirrhosis remains the second most common indication for liver transplantation. A comprehensive medical and psychosocial evaluation is needed when making a decision to place such patients on the transplant list. Most transplant centers worldwide need a minimum of 6 mo of alcohol abstinence for listing these patients. Patients with alcohol dependence are at high risk for relapse to alcohol use after transplantation (recidivism). These patients need to be identified and require alcohol rehabilitation treatment before transplantation. Recidivism to the level of harmful drinking is reported in about 15%-20% cases. Although, recurrent cirrhosis and graft loss from recidivism is rare, occurring in less than 5% of all alcoholic cirrhosis-related transplants, harmful drinking in the post-transplant pe-

riod does impact the long-term outcome. The development of metabolic syndrome with cardiovascular events and *de novo* malignancy are important contributors to non liver-related mortality amongst transplants for alcoholic liver disease. Surveillance protocols for earlier detection of *de novo* malignancy are needed to improve the long-term outcome. The need for a minimum of 6 mo of abstinence before listing makes transplant a nonviable option for patients with severe alcoholic hepatitis who do not respond to corticosteroids. Emerging data from retrospective and prospective studies has challenged the 6 mo rule, and beneficial effects of liver transplantation have been reported in select patients with a first episode of severe alcoholic hepatitis who are unresponsive to steroids.

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**Key words:** Alcoholic liver disease; Liver transplantation; Transplant evaluation; Recidivism; Six months rule; Alcoholic hepatitis

**Core tip:** Alcoholic cirrhosis remains the second most common indication for liver transplantation. Due to effective immune suppression regimens, graft loss and recurrent alcoholic liver disease rarely leads to mortality. However, the development of non-hepatic disorders such as malignancy and metabolic syndrome contributes to long-term morbidity and mortality. Although recidivism does impact long-term survival, data on the accuracy of 6 mo rule in predicting recidivism are scanty and controversial. Emerging data on the beneficial role of liver transplant provides a ray of hope for select patients with alcoholic hepatitis.

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## INTRODUCTION

In the United States, about 60% of the general population admits to alcohol use, and about 8%-10% report heavy drinking (2 or more drinks per day)<sup>[1]</sup>. One drink is equivalent to 12 oz. of beer (4%-5% weight by volume or w/v), 6 oz. of wine (8%-10% w/v), and 2 oz. of hard liquor or whiskey (45% w/v). Of the various factors responsible for liver disease, the most important are the duration and amount of alcohol consumed. Pooled data from several epidemiological studies report a minimum intake of 30 g/d of alcohol in women and 50 g/d in men, consumed over at least 5 years is required to cause liver cirrhosis<sup>[2]</sup>. The prevalence of and the mortality rates from cirrhosis parallel the prevalence rates of alcohol consumption in the population globally. Several host and environmental factors increase the risk of development of liver disease, such as gender (women are more prone), hard liquor compared to wine, binge drinking (5 or more drinks at one time), drinking on an empty stomach, genetic factors such as *PNPLA3* gene polymorphisms, concomitant hepatitis C virus (HCV) infection, and obesity<sup>[3,4]</sup>.

Alcohol remains the third most common preventable cause of death after smoking and hypertension. Alcohol-related mortality affects the young and middle-aged population, with loss of the most productive life years. Although cirrhosis is the fourth leading cause of death in the US in people aged between 45 and 54 years of age, the mortality rate rises with increasing age reaching as high as 31 per 100000 amongst people in the age group of 75-84 years. Liver-related complications from alcohol contributes to 4% in mortality and 5% in disability adjusted life years (DALY) globally with the highest impact in Europe where similar figures are 7% and 12% respectively<sup>[5]</sup>. This huge disease burden has an economic impact of about 125 billion Euros annually in Europe, accounting for 1.3% of the gross domestic product. In 2006, the estimated total economic cost of excessive alcohol consumption in the US amounted to \$223.5 billion. Of the \$223.5 billion, 72.3% (\$161.3 billion) represented the costs related to lost productivity, secondary to impaired productivity at work (45.9%) and lost productivity due to alcohol-related deaths (83180; 40.3%). In addition, another 11% (\$24.6 billion) is lost due to increased healthcare costs, the largest expenditures coming from specialty treatment for alcohol abuse and dependence (43.4%), and hospitalizations due to medical conditions related to excessive drinking (20.8%). The remainder of the expenditure was due to the costs associated with the criminal justice system (\$21 billion) and motor vehicle crashes (\$14 billion)<sup>[6]</sup>. These figures are probably underestimates due to inaccuracies in death certificate reports, since the mention of alcohol as contributing cause of death may have social and legal implications.

Alcohol abstinence remains the cornerstone in the management of patients with alcoholic liver disease with the potential for improvement in liver histology, com-

plications, and survival. However, complete reversibility of liver function usually does not occur once cirrhosis sets in. The use of corticosteroids and/or pentoxifylline in patients with severe alcoholic hepatitis provides about 50% survival benefit; nearly 20%-25% of such patients succumb to their illness despite treatment with these drugs. Thus, the curative management options for patients who are non-responsive to drugs and/or abstinence are limited, with the exception of liver transplantation (LT), a definitive treatment option for patients with cirrhosis and end-stage liver disease. In this article, we review the current status and special considerations on the use of LT for alcoholic cirrhosis and controversies and emerging data on liver transplantation for patients with severe alcoholic hepatitis that is non-responsive to pharmacological therapies.

## LIVER TRANSPLANTATION FOR ALCOHOLIC CIRRHOSIS

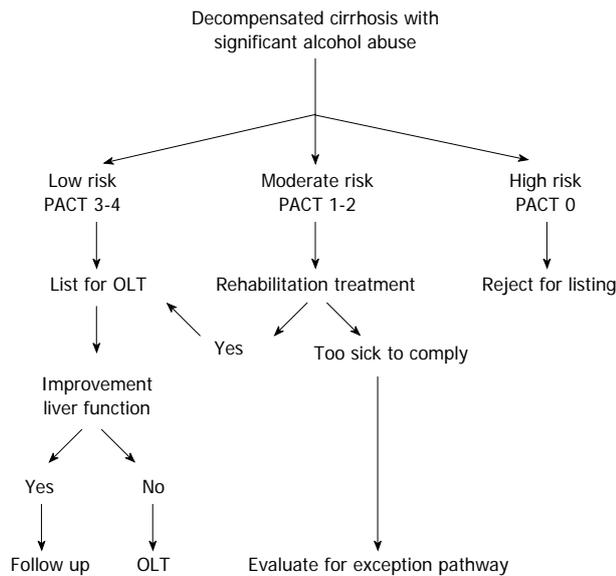
LT is a definitive treatment option for patients with cirrhosis and ESLD. Over the last two decades, advances in technical aspects of the operative procedure, intraoperative and postoperative care, and immunosuppression protocols have led to graft and patient survivals of over 90% at one year after LT<sup>[7,8]</sup>. Currently, LT is an accepted treatment modality for ESLD patients who have an estimated 1 year survival of less than 10%<sup>[9]</sup>. The excellent graft and patient outcomes of patients transplanted for alcoholic cirrhosis have encouraged physicians and the transplant communities to more readily refer these patients for liver transplant evaluation<sup>[8-10]</sup>. Alcoholic liver disease remains the second most common indication for LT, accounting for approximately 40% of all primary transplants in Europe and about 25% in the United States<sup>[8,11]</sup>. However, in spite of the encouraging data, disparities remain on the referral pattern of patients with alcoholic cirrhosis. In one study, only 21% patients were found to qualify for LT based on the current American Association for Study of Liver Diseases (AASLD) guidelines<sup>[12]</sup>.

### *Evaluation of alcoholic cirrhosis patients for liver transplantation*

Evaluation for LT of a patient with alcoholic liver disease requires a multidisciplinary approach, with important contributions from psychiatrists and addiction specialists. However, the overall evaluation process of a patient with alcoholic cirrhosis for LT is similar to that of a patient with non-alcoholic cirrhosis, including the indications and contraindications for LT. However, there are certain specific issues that need to be addressed during the evaluation process, which will be briefly discussed.

### *Evaluation for alcohol use*

The first step is to obtain a detailed and accurate history from the patient and the relatives and friends, of alcohol use to ascertain that the patient meets the criteria



**Figure 1** Psychosocial evaluation of patients with alcoholic cirrhosis awaiting listing for liver transplantation. OLT: Optical line terminal; PACT: Psychosocial Assessment of Candidacy for Transplantation.

for the diagnosis of alcohol-related cirrhosis<sup>[13]</sup>. Patients with alcohol dependence (tolerance to alcohol with use of increasing amounts, withdrawal symptoms, failed abstinence attempts, craving, and eye opener to avoid a hangover) should be identified, as these patients would often require rehabilitation treatment to maintain abstinence. As noted above, the history of alcohol use should be confirmed with relatives or friends since self-reported use of alcohol is often inaccurate. In one study, 18 of 82 alcoholic cirrhotic with a Department of Motor Vehicle record of driving under the influence (DUI) were not discovered on patient self-report<sup>[14]</sup>. Apart from the amount of alcohol use, it is mandatory to determine the date of the last drink in order to assess the duration of abstinence. A consensus conference in 1997 stated the need for minimum abstinence duration of 6 mo prior to listing a patient for LT (6 mo rule)<sup>[15]</sup>. The concept behind this rule was to allow the full benefit of abstinence on the liver functions, as studies have shown that the maximum benefit of abstinence is observed within the first 3-6 mo<sup>[16]</sup>. AASLD recommends that patients who continue to have significant liver disease despite 6 mo of abstinence should be considered and evaluated for LT<sup>[17]</sup>.

### Medical evaluation

A careful assessment should be made of the effects of alcohol on other organs including the presence of cardiomyopathy, chronic pancreatitis, Wernicke's encephalopathy, alcohol-related dementia, peripheral neuropathy, and upper aero-digestive malignancies as these can affect LT candidacy<sup>[18]</sup>. For example, patients with memory loss or confusion may be misdiagnosed as hepatic encephalopathy, instead of Wernicke's encephalopathy or alcohol dementia. Similarly, patients may have narcotic dependent due to chronic pancreatitis or may have poor

performance status due to peripheral neuropathy that is unrelated to the liver disease. These issues need to be addressed as they have a negative impact on LT outcome. Therefore, detailed cardiac and neurological assessment should be done in alcoholic cirrhotic to assure their LT candidacy. Although, malnutrition does not impact outcome after LT, malnourished patients are known to have greater length of hospital stay and consume more hospital resources compared to well-nourished patients<sup>[19]</sup>. Therefore, nutritional status should be assessed and malnourished patients should receive supplementation for optimizing their nutritional status.

### Psychosocial evaluation

Although psychosocial evaluation is mandatory for all transplant candidates, it is more important in alcoholic cirrhotic. Psychosocial Assessment of Candidacy for Transplantation (PACT) scale is a common tool used at most centers for evaluating candidates for all types of transplants<sup>[20]</sup>. This scale is used to assess social support, psychological health, life style factors, and patients' understanding of the transplant process including the follow up process after transplantation. Alcohol and substance abuse are only one part of this scale. Patients with intermediate risk for recidivism are recommended to undergo rehabilitation treatment before being considered for LT (Figure 1).

The data on the impact of rehabilitation treatment in maintaining abstinence are scanty, especially with respect to whether this treatment needs to be administered as out-patient sessions or as an in-patient intensive treatment for 2-3 wk followed by outpatient sessions. In a randomized study, motivational enhancement therapy (MET,  $n = 46$ ) was beneficial compared to treatment as usual (TAU,  $n = 45$ ) in reducing the amount and frequency of drinking prior to transplantation<sup>[21]</sup>. In this study, MET consisted of intensive 50-min sessions every month for 7 sessions along with Alcoholic Anonymous (AA) attendance. By contrast, TAU comprised of intensive outpatient treatment and community AA referral. However, compliance with the treatment was an issue since the average number of sessions in the MET group was only 3.8 instead of 7 as was proposed initially<sup>[21]</sup>. There are many reasons for failed adherence to rehabilitation treatment, the most important being inability to attend these sessions due to sickness from liver disease. These patients could be considered for exceptional pathway after multidisciplinary assessment and discussion (Figure 1). A randomized controlled trial on 84 alcoholic cirrhotic on the use of baclofen in maintaining abstinence and reducing hospital readmission rates has provided encouraging results<sup>[22]</sup>. It was observed that a higher proportion of patients maintained abstinence with baclofen compared to placebo (71% vs 29%,  $P = 0.0001$ ) and had a longer mean duration of abstinence (62 d vs 31 d,  $P = 0.001$ ). Further, there was improvement in liver function in patients treated with baclofen as compared to patients receiving placebo<sup>[23]</sup>.

**Detection of relapse to alcohol use (recidivism)**

While awaiting LT, patients should be followed regularly and closely to confirm abstinence as patients not complying with this recommendation should lose their listing status. At each visit, physicians should perform detailed history, urine drug screening, serum nicotine in recent or current smokers, and blood ethanol or other markers of alcohol use. In one study, random blood alcohol screens reduced recidivism by 37%<sup>[24]</sup>. Methanol testing is more sensitive and detects recent alcohol use when blood ethanol levels are normal<sup>[25]</sup>. Gamma glutamyltransferase (GGT) is a more sensitive test (70%) than aminotransferase for chronic heavy alcohol use. However, it is not specific in the pre-transplant setting as it may be elevated in cirrhotic patients<sup>[5]</sup>. Carbohydrate-deficient transferrin (CDT) is an FDA approved marker for alcohol abuse. Moderate to heavy alcohol use reduces the carbohydrate content of transferrin molecule synthesized by the liver<sup>[26]</sup>. Its performance in detecting alcohol use is better when combined with GGT, with sensitivity of 90% and 75% in males and females respectively<sup>[26]</sup>. However, since CDT levels are impacted by the severity of liver disease, its use is limited in the pre-transplant setting. Further, CDT levels are affected by smoking and need to be adjusted for total transferrin levels in females<sup>[27]</sup>. New biomarkers using metabolites of alcohol such as ethyl glucuronide (eTG) are under investigation. Urine eTG is extremely sensitive and can detect alcohol level as low as 10 ng/mL and may become falsely positive with the use of alcohol-containing medications and hand sanitizers. In one study, a cut-off level of 0.5 mg/L, as detected by liquid chromatography-mass spectroscopy (LC-MS), is 89% sensitive and 99% specific with a negative predictive value of 99% and positive predictive value of 89%. Increasing the cut-off level to 1 mg/L did not improve the specificity but decreased the sensitivity to 75%<sup>[28]</sup>. In this study, combining UeTG with CDT could accurately diagnose 93% of patients with recidivism. UeTG may be falsely negative in patients with urine infection especially with *E. coli* as this metabolite of alcohol may be degraded by the bacterium<sup>[29]</sup>. Ethyl sulfate (EtS) another metabolite of alcohol is not affected by bacterial degradation and its simultaneous measurement in the urine can overcome this limitation<sup>[30]</sup>. However, the short-term window for positivity limits the use of these markers in routine clinical practice after the last heavy alcohol use: a few hours for BAL and methanol, and 4-5 d for UeTG and EtS<sup>[28]</sup>. In this regard, hair analysis for ethyl glucuronide is useful since the metabolite remains in hairs for one month<sup>[31]</sup>. A cut off level of 7 pg/mg of hair sample is a strong indicator of regular alcohol use, and a cut off level of 30 pg/mg can be accurately diagnose heavy drinking<sup>[32]</sup>. Other markers such as phosphatidyl ethanol, acetaldehyde, Cytochrome P-450 (CYP) E, mono amino oxidase-B, 5-tryptophol, and fatty acid ethyl ester are experimental and unavailable for clinical application at the present time<sup>[33]</sup>.

**Recidivism after liver transplantation for alcoholic cirrhosis**

Recidivism is reported in 10%-60% of transplant recipients for alcoholic liver disease<sup>[34-37]</sup>. The large variation in prevalence rates of recidivism is perhaps due to different definitions used, with some defining recidivism as any alcohol use while others only include harmful drinking, defined as 2 or more drinks per day, which is reported in 15%-20% patients. Recidivism rates across different studies are also affected by the duration of follow up. In a pooled analysis from 50 studies evaluating recidivism after liver transplantation, the rates of any alcohol use after LT were 5.7% per 100 person years, and 2.5% per 100 person years risk for harmful drinking<sup>[38]</sup>. DiMartini *et al.*<sup>[39]</sup> reported on the patterns of alcohol use after LT. Nearly 45% patients reporting some alcohol use (26% rare slips and 19% harmful drinking). Of those with harmful drinking, about 1/3<sup>rd</sup> each reported early start (within 2-3 years after LT) with subsequent decline, continued harmful drinking throughout, and late start (after the first 3 years and then persisting with alcohol use).

Data on the accuracy of 6-mo pre-transplant abstinence in predicting recidivism are scanty and controversial. In a systematic review of 22 studies reporting predictors of recidivism, only 2 of the 11 studies evaluating this variable reported it to be an accurate predictor of recidivism<sup>[40]</sup>. Social and family support, preexisting psychiatric comorbidities, polysubstance use, unsuccessful attempts at rehabilitation, younger age at LT, and family history of alcoholism emerged as the strongest predictors for recidivism<sup>[40]</sup>. In another study based on the duration of drinking, the number of daily drinks consumed, and the number of previous alcoholism treatments, the high-risk alcoholism relapse (HRAR) score (ranging from 0-6) was calculated and compared with 6-mo abstinence in predicting recidivism. HRAR emerged as a stronger predictor of abstinence with 79%, 69%, and 54% agreement between HRAR and 6 mo abstinence for low, moderate, and high HRAR groups respectively<sup>[41]</sup>. The impact of pre-transplant motivational enhancement therapy on recidivism could not be assessed in a randomized study due to the small sample size<sup>[21]</sup>.

**Outcomes of patients transplanted for alcoholic cirrhosis**

The patient survival rates after LT for alcoholic cirrhosis based on data from different parts of the world have been reported to be 81%-92%, 78%-86%, and 73%-86% at 1, 3, and 5 years respectively<sup>[8,11,42]</sup>. There is improvement in the quality of life, mood status and cognitive functioning, with no difference compared to patients transplanted for non-alcoholic cirrhosis<sup>[43,44]</sup>. Patients were able to return to society and lead active and prolific lives, irrespective of the indication for transplantation<sup>[34,45]</sup>.

Concomitant alcohol abuse and HCV infection is observed in about 14% of individual with chronic liver disease<sup>[3]</sup>. The data on outcomes in HCV positive drink-

**Table 1** Studies on recidivism and its impact on liver graft and liver related death

Ref.	Total No.	Median FU year	Percent recidivism	Percent graft loss alcohol related	LRD as percent of all deaths
Conjeevaram <i>et al</i> <sup>[55]</sup>	68	3.5	8	38	38
Cuadrado <i>et al</i> <sup>[52]</sup>	99	8.25	26	0	NR
Pageaux <i>et al</i> <sup>[94]</sup>	121	4.5	21	1	12
Lucey <i>et al</i> <sup>[35]</sup>	50	5.25	33 <sup>1</sup>	6	6
Pfizzmann <i>et al</i> <sup>[95]</sup>	300	7.5	7	50	88
Schmeding <i>et al</i> <sup>[51]</sup>	271	10	27	0	NR
Dumortier <i>et al</i> <sup>[78]</sup>	305	5.25	12	2	8

<sup>1</sup>Any use of alcohol in this study was reported as recidivism. Other studies defined recidivism as heavy drinking defined with variable amount of alcohol use across different studies. LRD: Liver related death.

ers are scanty and conflicting. Studies using transplant registries from the United States and Europe, suggest worse survival outcomes compared to HCV negative alcoholic cirrhosis<sup>[11,46,47]</sup>, although a study from a single European center reported similar outcomes<sup>[48]</sup>. It should be noted that in the latter study, patients received antiviral therapy more often compared to patients with HCV cirrhosis alone, which may explain the difference in the outcome<sup>[48]</sup>. Unfortunately, such information is lacking in data collected from registries. Further, HCV positive drinkers may have been misclassified in the registry databases due to lack of information on the amount of alcohol use. Further studies using data from single or multiple centers with detailed information on these variables are needed to examine post-transplant outcomes of alcoholic cirrhosis and concomitant HCV infection.

Relapse to harmful drinking affects long-term patient survival. Compared to abstinent patients transplanted for non-alcoholic liver diseases, the survival rates in patients with recidivism to harmful drinking are similar initially but become worse after 5-10 years (45%-68% *vs* 75%-86%)<sup>[49-52]</sup>. The proportion of patients dying from liver-related cause in patients transplanted for alcoholic cirrhosis varies from 6%-88% in various series (Table 1). Graft loss from recurrent disease related to alcohol use is rare<sup>[53,54]</sup>. Rates of graft loss due to recidivism are 0%-6% in most series except two studies from the same institution which reported 38% and 50% graft loss related to alcohol use (Table 1)<sup>[55,55]</sup>. Harmful drinking in the early phase of post-transplant period is more significant in terms of the impact on liver graft. In one study, graft loss and liver-related mortality occurred in 72% and 45% respectively in patients with early onset harmful drinking compared to 45% and 0% amongst those who were abstinent, had rare slips, or later use of harmful drinking<sup>[56]</sup>. Recurrent cirrhosis and graft loss accounts for about 3% of the original cohort of transplant recipients for alcoholic cirrhosis<sup>[57]</sup>. It is unclear whether certain genetic factors in the donor liver play a protective role despite recidivism to harmful drinking in these patients who were originally genetically predisposed to alcohol-related liver injury. However, once recurrent cirrhosis sets in, the outcome is worse compared to patients with a functional graft (30%  $\pm$  17% *vs* 75%  $\pm$  6% survival at 10 years,  $P = 0.045$ )<sup>[57]</sup>.

With the introduction of effective immune suppression regimens, which protect the liver graft, non-hepatic disorders including metabolic syndrome and malignancies have become more important causes of patient mortality after LT for alcoholic liver disease. Registry analyses from Europe and United States have shown that cardiovascular causes and *de novo* malignancies were significantly over-represented in patients who had undergone transplantation for ALD *vs* recipients without ALD<sup>[11,47]</sup>. Although, the metabolic syndrome is seen frequently on long term follow up after LT, patients transplanted for alcoholic cirrhosis are particularly prone to this complication followed by patients transplanted for non-alcoholic steatohepatitis-related ESLD<sup>[58,59]</sup>. The development of the metabolic syndrome is a risk factor for cardiovascular death in patients who survive the first year after LT<sup>[60]</sup>.

The development of *de novo* malignancy in LT recipients was recognized as early as 1972<sup>[61]</sup>. About 5%-15% of patients receiving liver transplantation develop *de novo* extrahepatic malignancy. Skin cancer accounts for 30%-50% followed by post-transplant lymphoproliferative disorders (PTLD) and solid organ cancers<sup>[62,63]</sup>. The risk is higher compared to the general population for skin as well as solid organ cancers with standardized incidence ratio of about 15 and 2-2.5 respectively<sup>[64,65]</sup>. The risk increases with time to as high as 19% and 34% at 10 and 15 years post-transplant respectively<sup>[64]</sup>. The risk for *de novo* malignancy is 1.5-2 folds higher in transplant recipients for alcoholic liver disease compared to transplants for non-alcohol-related etiologies<sup>[66-69]</sup>. Patients transplanted for alcoholic cirrhosis are at a unique risk for the development of upper aero-digestive cancers with about 10-fold higher risk compared to transplants for other indications<sup>[68-70]</sup>. Intensive screening for head and neck cancers prior to transplant does not seem to be cost effective, with only 0.17% prevalence of this cancer in one study that evaluated 581 patients with alcohol-related cirrhosis<sup>[71]</sup>.

The use of immune suppression post-transplantation is believed to be the major mechanism for the development of *de novo* malignancy<sup>[62,63]</sup>. Other risk factors are older age, male gender, and Epstein Barr virus reactivation or infection for lymphoproliferative malignancy, and exposure to sun for non-melanoma skin cancer<sup>[63,72,73]</sup>. The mechanisms involved in predisposing alcohol-related

transplant patients to malignancy are poorly understood. Oncogenic properties of acetaldehyde, a metabolite of alcohol, and the inhibition of DNA methylation have been blamed<sup>[74]</sup>. Smoking both pre and post-transplant increases the risk for upper aerodigestive cancers in patients transplanted for alcoholic cirrhosis<sup>[62,63]</sup>. In one study, 60% of the 202 LT recipients analyzed reported a life time history of smoking, with 54% using both alcohol and tobacco in the pre-transplant period. Of those who quit, 20% patients had a relapse to smoking in the post-LT period<sup>[75]</sup>. In another study, a higher proportion of transplanted patients had a positive smoking history compared to transplants for non-alcoholic diseases (82% *vs* 45%,  $P = 0.001$ ), with a higher mean number of cigarettes smoked by alcoholics (25 *vs* 16 cigarettes per day,  $P = 0.001$ )<sup>[76]</sup>.

The development of malignancy has a significant impact on patient survival, with about 38% and 53% risk of death at 1 and 5 years after diagnosis<sup>[68]</sup>. *De novo* malignancy accounts for 30%-40% of all deaths in LT recipients who survive the first year after transplantation<sup>[77,78]</sup>. Implementation of intensive surveillance protocols in the post-transplant period has been shown to improve survival by detection of these cancers at an early stage<sup>[79,80]</sup>. Patients should be instructed to use sun screens when exposed to sun, come for annual physical checkup including skin and ENT examinations, and avoid use of nicotine and alcohol. Intensive protocols have included annual chest X-ray, urine examination, CT chest, abdomen and pelvis, mammography, PAP smear, in addition to standard guidelines for colonoscopy screening. With such a protocol, the overall survival in the surveillance group showed significant improvement (11.3 years *vs* 3.1 years,  $P = 0.001$ )<sup>[79]</sup>. However, clear guidelines for other cancers including the frequency of work-up have not been developed.

A higher occurrence of neurological complications has been reported in patients transplanted for alcoholic liver disease, resulting in greater resource utilization<sup>[81,82]</sup>. These include profound confusion in the early postoperative period, and structural injury from prolonged alcohol use which remained unrecognized before transplant<sup>[83,84]</sup>. Therefore, detailed and accurate pre-transplant assessment for neurological issues is needed in patients with alcoholic-related cirrhosis, as alluded to earlier in the pre-transplant evaluation section.

## LIVER TRANSPLANTATION FOR ALCOHOLIC HEPATITIS

Alcoholic hepatitis is a distinct clinical syndrome seen in patients with chronic and active alcohol use with a potential for mortality of 40%-50% in patients with untreated severe disease. Alcoholic hepatitis occurs in 35%-40% of patients with chronic excessive alcohol use, and represents about 0.2% (20 of every 1000) hospital admissions in the United States<sup>[85,86]</sup>. The true prevalence is difficult to assess as many patients remain undiagnosed and only

10%-35% of alcohol-related cirrhotic may have changes consistent with alcoholic hepatitis on liver biopsy<sup>[87]</sup>. The incidence rates of ALD-related deaths decreased from 6.9/100000 persons in 1980 to 4.4/100000 persons in 2003. The age- and sex-adjusted alcoholic liver disease related mortality (per 100000 persons) decreased from 6.3 to 4.5 in Caucasians, 11.6 to 4.1 in African Americans, and 8.0 to 3.7 in the "other" race groups<sup>[88]</sup>. Overall, the incidence of alcoholic hepatitis is about 7%-10% in mild illness and 40%-50% with severe disease<sup>[85-89]</sup>. Although, mortality from alcoholic hepatitis has decreased over the last decade as with alcoholic cirrhosis, patients with alcoholic hepatitis and concomitant HCV infection remain at a higher risk of death with 20%-25% higher mortality as compared to alcoholic hepatitis patients without HCV infection<sup>[86]</sup>. One of the possible reasons may be fear of physicians and gastroenterologists in using corticosteroids for the treatment of alcoholic hepatitis in the presence of HCV infection<sup>[90]</sup>. More studies are needed aimed at generating guidelines for managing alcoholic hepatitis patients who also have HCV infection.

The available treatment options that include the use of corticosteroids and/or pentoxifylline achieve about 50% survival benefit, with the likelihood of mortality in about 20%-25% patients<sup>[85,89]</sup>. With the current lack of available pharmacological options for patients with non-response to steroids, there remains an unmet need for the development of newer and more effective drugs. These patients generally do not qualify for LT because of the requirement of 6 mo of abstinence demanded by most transplant centers worldwide. This requirement cannot be met by alcoholic hepatitis patients who do not respond to steroids since by definition they were drinking up until at least 3 wk prior to getting sick. Further, there are other ethical concerns and controversies involved in the use of LT for these patients such as: (1) public opinion that this disease is self-inflicted; (2) shortage of donor livers and the view that they should be allocated to more deserving patients; and (3) the risk of recidivism after LT<sup>[91]</sup>. The other side of the argument is that pre-transplant abstinence of 6 mo is not a strong predictor of recidivism. Further, patients transplanted for alcoholic cirrhosis with histological changes consistent with alcoholic hepatitis on explants have been shown to have similar post-LT survival compared to patients without such histological changes<sup>[87]</sup>. An obvious ethical question is whether severe alcoholic hepatitis patients who do not respond to treatment should be left to their fate to die or should be considered for LT as suggested by the French consensus group<sup>[91]</sup>.

The same group took the lead and challenged the 6 mo abstinence rule in a prospective case control study. In this study, 26 patients (mean age 47 years, 58% males) with a first episode of severe alcoholic hepatitis (median MELD 30<sup>[22-47]</sup>) who did not respond to steroids were recruited from four different centers in Europe (2006-2010) and received LT as a life-saving option. The patients underwent detailed and rigorous psychosocial evalua-

tion by the resident team, hepatologist, anesthesiologist, and surgeon and LT was approved only if all four teams cleared the patient. The median duration between declaring a patient as non-responder to steroids (Lille score  $\geq 0.45$ ) and receipt of LT was 9 (1-13) d<sup>[92]</sup>. Compared to the 26 matched patients who did not receive LT (control group), patients receiving LT had a significantly better outcome at 1 mo (77%  $\pm$  9% *vs* 25%  $\pm$  8%,  $P < 0.001$ ) and at 2 years (73%  $\pm$  8% *vs* 23%  $\pm$  8%,  $P < 0.001$ ). Patients receiving LT had survival similar to patients responding to steroid treatment (78%  $\pm$  8% *vs* 83%  $\pm$  4%,  $P = 0.33$ )<sup>[92]</sup>. As can be seen, the majority of deaths in the control group occurred within the first month, indicating the importance of transplanting these patients early without waiting for the 6 mo abstinence requirement. Three patients resumed alcohol intake at 720, 740, and 1145 d after LT. The first two patients reported harmful drinking of 30 g/d and  $> 50$  g/d of alcohol respectively, while the 3<sup>rd</sup> patient was consuming a lower amount, about 10 g/wk. However, recidivism in this study was self-reported which is known to be frequently inaccurate<sup>[92]</sup>. None of the patients lost the graft due to alcohol use. However, three of the 6 deaths in this study were due to invasive fungal infections. This is unlikely to be due to pre-existing infections as all these patients were undergoing daily rigorous infection work-up prior to LT. Prospective studies are required to evaluate strategies of immune suppression for preventing fungal infections. The authors concluded that LT should be considered as a salvage option in select patients with severe alcoholic hepatitis who do not respond to steroids. With the strict selection of patients, only about 2%-3% of the original cohort with alcoholic hepatitis was amenable to this treatment modality<sup>[92]</sup>. In the United States, nearly 50000 patients with alcoholic hepatitis are admitted annually. Considering that about 20% of these patients have severe disease, which translates to about 200-300 patients who may qualify for LT as a therapeutic option. However, any amendment in the guidelines for liver transplantation in patients with alcoholism may have an adverse impact on public preferences for liver-transplant allotment and may decrease willingness to donate. However, this has not occurred in response to transplantation being offered to patients with fulminant hepatic failure due to self-inflicted acetaminophen poisoning, or in intravenous-drug users with acute hepatitis B virus infection. Before implementing this in routine practice, with the potential of adversely affecting the liver donor pool, more data are needed on larger patient populations. Until then, there is a ray of hope for patients with a first episode of alcoholic hepatitis who do not respond to steroids and have excellent psychosocial support systems.

Similar findings were reported in a retrospective study using the UNOS database. In this study, 55 patients (mean age 51 years, 76% males) received LT (2004-2010) for a listing diagnosis of alcoholic hepatitis (median MELD 24<sup>[16-34]</sup>). The results were compared with 165 patients (matched for age, gender, UNOS region, MELD score,

and donor risk index), transplanted for alcoholic cirrhosis. There was no difference in the respective outcomes at 1, 3, and 5 years for liver graft survival (87% *vs* 84%,  $P = 0.58$ , 82% *vs* 77%,  $P = 0.47$ , and 75% *vs* 73%,  $P = 0.97$ ) and patient survival (93% *vs* 88%;  $P = 0.33$ , 87% *vs* 81%,  $P = 0.33$ , and 80% *vs* 78%,  $P = 0.91$ )<sup>[93]</sup>. A higher proportion of alcoholic hepatitis patients had concomitant HCV infection compared to the alcoholic cirrhosis controls (27% *vs* 7%,  $P = 0.02$ ). After controlling for HCV and other recipient and donor factors, the graft and patient survival was not different, with OR (95%CI) of 0.82 (0.41-1.63) and 0.7 (0.33-1.54) respectively<sup>[93]</sup>. Since patients with alcoholic hepatitis have underlying alcoholic cirrhosis in about 60%-70% cases, the outcomes were also compared based on the explant diagnosis. A total of 46 patients with an explant diagnosis of alcoholic hepatitis were compared with 138 patients with an explant diagnosis of alcoholic cirrhosis, again the outcomes were similar. Moreover, 11 patients with a listing as well as explant diagnosis of alcoholic hepatitis compared to 33 patients with a listing and explant diagnosis of alcoholic cirrhosis had equivalent outcomes<sup>[93]</sup>. None of the patients died or lost their graft secondary to alcohol use. However, in addition to the drawbacks of a retrospective study, this study was limited by the lack of information on alcohol drinking history.

## SUMMARY AND FUTURE PERSPECTIVES

In conclusion, alcoholic cirrhosis remains the second most common indication for LT. Although, outcomes are excellent in general, recidivism does impact long-term survival. Graft loss and recurrent alcoholic liver disease rarely leads to mortality. However, the development of metabolic syndrome and *de novo* malignancy contribute to the majority of deaths in the long term. The emerging data on the beneficial role of LT provides a ray of hope for select patients with alcoholic hepatitis with the following characteristics: (1) first episode of severe alcoholic hepatitis; (2) failure to respond to pharmacological approach; and (3) excellent psychosocial support. However, certain unsettled issues need to be resolved. These include: (1) The current discrimination for transplant evaluation and transplant listing of patients with alcoholic cirrhosis; (2) physician and center based barriers to liver transplant evaluation and listing; (3) accurate predictors independent of pre-transplant sobriety duration for post-transplant relapse to heavy drinking; (4) genetic factors in the donor graft that may protect the recipient from recurrent disease despite relapse to heavy drinking; (5) simple and accurate biomarkers for use in clinical practice to detect recidivism with a long window period; (6) cost-effective surveillance protocols for early detection of *de novo* malignancy after liver transplantation; (7) creation of a prospective database on the outcome of LT in patients with a first episode of acute alcoholic hepatitis; and (8) development of better immune suppression regimens in these patients, especially

in reducing invasive fungal infections.

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## How reliable is current imaging in restaging rectal cancer after neoadjuvant therapy?

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60% in most studies, both for rectal wall invasion and for lymph nodes involvement. In particular the ability to predict complete pathological response, in order to tailor the surgical approach, remains low. Due to the radio-induced tissue modifications, combined with imaging technical aspects, low rate accuracy is achieved, making modern imaging techniques still unreliable in restaging rectal cancer after chemo-radiotherapy.

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**Key words:** Rectal cancer; Restaging; Neoadjuvant therapies; Diagnostic accuracy; Complete pathological response

### Abstract

In patients with advanced rectal cancer, neoadjuvant chemo radiotherapy provides tumor downstaging and downsizing and complete pathological response in up to 30% of cases. After proctectomy complete pathological response is associated with low rates of local recurrence and excellent long term survival. Several authors claim a less invasive surgery or a non operative policy in patients with partial or clinical complete response respectively, however to identify patients with true complete pathological response before surgical resection remains a challenge. Current imaging techniques have been reported to be highly accurate in the primary staging of rectal cancer, however neoadjuvant therapy course produces deep modifications on cancer tissue and on surrounding structures such as overgrowth fibrosis, deep stroma alteration, wall thickness, muscle disarrangement, tumor necrosis, calcification, and inflammatory infiltration. As a result, the same imaging techniques, when used for restaging, are far less accurate. Local tumor extent may be overestimated or underestimated. The diagnostic accuracy of clinical examination, rectal ultrasound, computed tomography, magnetic resonance imaging, and positron emission tomography using 18F-fluoro-2'-deoxy-D-glucose ranges between 25% and 75% being less than

**Core tip:** Neoadjuvant chemoradiotherapy has become the standard treatment for patients with advanced rectal cancer allowing reduction of local recurrences and increased sphincters' preservation. New trends have proposed the possibility to change the planned surgical resection after neoadjuvant treatment, in case of extensive tumor response, and several Authors claim limited resection or non operative "wait and see" policy. In this setting restaging plays a crucial role in identifying patients with complete response. The diagnostic accuracy in predicting tumor response of the currently available imaging techniques is extensively reviewed in order to determine the reliability.

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### INTRODUCTION

In the last two decades the treatment of rectal cancer has been modified by virtue of the introduction of neoadju-

vant treatment<sup>[1]</sup>, better imaging techniques and improvement of surgery with total mesorectal excision (TME). The crucial goals reached by upgrading diagnostic techniques and therapeutic strategies accounts for reduction of local recurrence rate and increase of sphincter preserving surgeries.

Preoperative chemoradiation therapy (CRT) has become the standard treatment in the last decade<sup>[1-3]</sup>. Advantage of neoadjuvant treatment is the downsizing/downstaging of the tumor thus allowing the preservation of the sphincter, in case of extremely distal rectal lesions, and, often, avoiding multiorgan resection in case of responding tumor that had involved other organs before the neoadjuvant regimen. The overall benefits of this therapeutic regimen are, eventually, the reduction rates of local recurrence and the improvement in survival<sup>[4-7]</sup>.

Reduction of local recurrence after surgery was first achieved with the introduction of complete excision of the visceral rectal mesentery, bringing up the concept that mesorectum harbors positive lymph nodes and tumor residues<sup>[8]</sup>. Moreover, it has been highlighted that surgical local radicality has to be carried out by improving the control on radial margins tumor-spread other than distal and proximal ones<sup>[9,10]</sup>. These findings have been demonstrated by pathological analysis of circumferential resection margin (CRM).

The key of neoadjuvant treatment and restaging of the tumor is, finally, the possibility of changing the planned surgical treatment and, in particular, the chance of providing a sphincter preserving procedure. In a more experimental way, new trends have proposed local resection in case of extensive tumor response<sup>[11]</sup> or a non-surgical “wait-and-see course” in case of complete tumor regression<sup>[12-15]</sup>. On the contrary, for non-responding or poor responding rectal tumors, more aggressive, traditional surgery, after restaging, is indicated. This decision depends mostly on the reliability of the imaging techniques provided by modern technology and the synergy between the radiologist and surgeon.

The main issue of re-staging after CRT with imaging techniques, is to discriminate cancerous mass from non malignant tissue because of the radio induced overgrowth fibrosis<sup>[16]</sup>. Tumor tissue changes, during and within 6-8 wk after chemo-radiotherapy, account for deep stroma alteration. Fibrosis compresses the colon tissue and ends up in causing wall thickness and muscle disarrangement. Other variation are tumor necrosis, calcification, and inflammatory infiltration of lymphocytes and macrophages<sup>[17]</sup>.

The tumor regression grade (TRG) exactly reflects the ratio between residual tumor percentage and overgrowing fibrosis percentage. Thus the more reliable restaging technique has, eventually, the goal to predict TRG because it positively correlates with disease free and overall survival<sup>[18]</sup>. Ideally, precise staging of rectal cancer has to define the tumor depth of invasion through the rectal wall, detect positive lymphnodes, and establish the resectability of locally advanced tumors.

Several techniques have been described to restage rectal carcinoma after CRT, the most predominantly used being computer tomography (CT) scan, rectal ultrasounds (RUS), and magnetic resonance imaging (MRI).

In the present study we revise the accuracy and reliability of current techniques, used to re-stage rectal cancer after neoadjuvant therapy, in terms of sensitivity, specificity, and diagnostic accuracy, compared with pathological findings after surgical resection. Special attention will be paid to the ability to predict complete response (cPR).

## CLINICAL EXAMINATION

There is no doubt that clinical examination, comprising digital rectal examination (DRE) and proctoscopy, is the first and essential approach to patients with rectal cancer. Moreover, clinical assessment of response to CRT may provide important information regarding the surgical strategy. Nevertheless few studies evaluated the accuracy of clinical assessment in predicting tumor response after completion of CRT, and the majority of these studies were retrospective. Clinical assessment may underestimate<sup>[19,20]</sup> or overestimate<sup>[21]</sup> pathological response therefore most authors claim that clinical examination is inaccurate and should not be used as the unique mean to define the efficacy of neoadjuvant therapy.

Only 2 studies tried to answer the question whether clinical parameters are able to predict cPR. In the study by Perez *et al*<sup>[22]</sup>, 99 patients were prospectively examined, by the same experienced colorectal surgeon, after 12 wk from completion of CRT; 16 patients had a complete clinical response (cCR), 3 underwent local excision of a residual scar, and a cPR was confirmed; 13 patients were enrolled in a strict follow-up without radical surgery, only one patients subsequently developed a local recurrence after a mean follow-up of 42 months; moreover the cCR positively correlated with the PET results<sup>[22]</sup>. On the contrary Hiotis *et al*<sup>[23]</sup> retrospectively analyzed 448 patients and found that 75% of patients with cCR had residual cancer in the resected specimens: 60% having T2 or T3 disease, and 18% node-positive disease. In addition, in the group of patients with no residual primary tumor at histology (T0), the percentage of node positivity was 15%.

Habr-Gama *et al*<sup>[24]</sup>, in an effort to standardize the clinical findings, clearly defined clinical and endoscopic sign to define complete response as: whitening of the mucosa, with or without telangiectasia, or loss of pliability of the rectal mucosa, absence of deep or superficial ulceration, or palpable nodule or stenosis located in previously tumor bearing area. Nevertheless the likelihood of detecting occult nodal disease in patients with no residual primary tumor is highly unlikely.

In conclusion even if clinical parameters may predict tumor response, they are unable to distinguish cPR and to predict which patient does not require surgical excision following CRT.

## RECTAL ULTRASOUNDS

The assessment of rectal tumor by means of ultrasonography is based on the evaluation of depth of invasion through the 5 layers of the bowel wall. With high resolution probes T1-2 tumors can be correctly diagnosed and even SM1, SM2 and SM3 tumors can be recognized<sup>[25]</sup>. On the other hand the mesorectum and peritoneum cannot be visualized by endorectal probe thus limiting the use of ultrasound for the evaluation of CRM.

Endorectal ultrasound can visualize perirectal lymph nodes and nodes located in the mesorectum while lymph nodes along the mesentery or the upper pelvis are generally unreachable. Normal lymph nodes are usually not seen sonographically. Enlarged lymph nodes are considered benign if oval shaped, and thought to be inflammatory if hyper echoic with well defined margins. There is no agreement as when to consider a lymph node as pathological. Dimensional, morphologic, and echographic pattern are to be considered. Nodes greater than 5 mm, round shaped and with echogenicity similar to the primary tumor (hypoechoic), are usually considered as predictors of metastatic involvement by most, but not all authors, who may choose only one of the aforementioned parameters, in particular size is considered the most reliable feature.

Different probes are employed for imaging rectal cancer including transrectal rigid, rotating or non rotating probe, either two-dimensional or with three-dimensional reconstruction, flexible endoscope either radial or linear, miniprobes able to pass through the biopsy channel<sup>[26]</sup>, with frequency ranging between 5 and 10 MHz. No comparison of the performance of different instruments has been made up to now. Unlike other imaging modalities, the endorectal ultrasound in the different settings is performed by a radiologist, a gastroenterologist or a colorectal surgeon, and this could be a further confounding factor when examining the accuracy of the examination.

There is no consensus regarding the time that must elapse between CRT and evaluation. The majority of the authors re-examined the patients after 4 to 6 wk. However a better diagnostic accuracy for N staging has been reported by Huh when the patients are re-evaluated after 7 wk<sup>[27]</sup> from completion of CRT.

Accuracy in T restaging ranges between 27% to 72%, with overstaging between 16% and 53%. In the majority of the studies T1-2 stage are more misdiagnosed than T3<sup>[8,9,11-13,16,18,28-30]</sup>. When examining the accuracy to correctly diagnose T0 the figure drop to 0% to 60%<sup>[31-33]</sup>. Gavioli *et al*<sup>[29]</sup> studied the modification of morphology induced by radiotherapy in 29 patients. They found that fibrosis replacing tumor corresponded to hypoechoic pattern at ultrasound that was difficult to differentiate from the pattern related to the tumor itself, thus inducing overstaging. In some cases of complete pathological response the fibrosis caused persistent interruption of the 5 layers leading to misinterpretation of the examination.

The accuracy in restaging lymph nodal involvement is somehow higher than accuracy for primary tumor, ranging between 39% and 83% and being around 70% in most studies. For this parameter overstaging was only slightly more common than understaging (8%-39% *vs* 11%-28%). Correct identification of N0 varies between 70% and 80%<sup>[8,9,11-13,16,18,28-30,34]</sup>. Moreover 13%-55% of patients with lymph nodal involvement were recognized as N0<sup>[30,34,35]</sup>.

When compared with other imaging techniques, namely CT and standard MRI, ultrasound resulted the most accurate in determining rectal wall infiltration and lymph node involvement in some studies<sup>[31]</sup>, while performed worse in others<sup>[27]</sup>.

It is of note that in the majority of the studies diagnostic accuracy is reported separately for T and N stage, thus preventing accuracy for complete pathological response (ypT0N0) to be determined. Kahn examined 25 patients with T0N0 tumors after preoperative radiotherapy and reported that endorectal ultrasound failed to detect the absence of disease in 83% of patients, with overstaging of T0 lesions diagnosed as T1 in 67% of cases and T2 in 16%. In the 25 patients' series of Maor, ultrasound correctly predicted postchemoradiation T0N0 stage in only 50% of cases. Radovanovic reported only one correct diagnosis out of 5 patients (20%) with cPR<sup>[36]</sup>. While complete remission was not correctly predicted in any of the 11 patients by Huh *et al*<sup>[27]</sup>.

The occurrence of uT0 harboring microscopic foci of tumor at histology is also reported<sup>[35]</sup>.

In conclusion endorectal ultrasound is insufficient in detecting which tumors become T0N0 after neoadjuvant treatment to possibly undergo limited resection or non-operative treatment.

## COMPUTER TOMOGRAPHY

CT is one of the preferred tools to evaluate tumor response, in relation to the tumor size modification, because of its high reproducibility and availability. Compared with the other commonly used techniques, CT scan is more largely accessible, faster, inexpensive and less operator-dependent. Also the unique advantage of CT is that a single scan provides staging for local tumor and distant metastasis. Therefore every re-staging techniques pattern usually includes a total body CT scanning.

Accuracy of CT scan in predicting T stage after neoadjuvant course is still debated in the literature and the results are often inconsistent or discordant<sup>[17,27,37]</sup>.

In a recent study, 90 patients with locally advanced rectal cancer were prospectively analyzed before and after neoadjuvant regimen. Accuracy of CT in predicting pathological T after radiotherapy was low (37%). However CT was reported to be accurate in the identification of involved CRM (71%)<sup>[37]</sup>. Conversely, Lee *et al*<sup>[17]</sup> have demonstrated, in a series of 91 patients undergoing CT restaging after neoadjuvant course, that T status positively correlated with pathological examination with an accu-

cy of 61%. Moreover they found a statistically significant correlation with CT downstaging assessment and TRG at pathology. However over staging was frequently found in patients with fibrosis and alteration in muscle disarrangement<sup>[17]</sup>. In the study conducted by Huh *et al*<sup>[27]</sup> on 80 patients, CT accuracy in restaging the depth of rectal wall invasion was poor (46.3%). CT was also found to more likely overstage T3 tumor and understage T2 ones<sup>[27]</sup>.

Finally CT scan is commonly considered an unreliable restaging technique to assess cPR<sup>[17,38]</sup>. In none of patients retrospectively analyzed by Huh CT scan was able to predict cPR<sup>[27]</sup>.

Nodal involvement detection plays a crucial role in those selected cases which are candidates to receive a local excision after extensive tumor response. In a local excision setting, it is compulsory to be aware of any residual nodal disease risk. Moreover the size of lymph nodes “per se” is considered not satisfactory for the determination of presence of disease. It has been shown that also texture arrangement and nodes profile are prognostic factors for malignancy<sup>[39,40]</sup>. However restaging lymph nodes after neoadjuvant course could also be more complex since radiotherapy has the ability to reshape and modify the size and the texture of the nodes.

In terms of nodal involvement CT has an accuracy of 82% by using a cut off of 10 mm<sup>[37]</sup>. On the contrary, in a 5mm cut-off setting, accuracy has been reported to be 62%<sup>[38]</sup>. In Huh series, with respect to nodal involvement, CT demonstrated a sensitivity of 56% and a specificity of 74%.

## MAGNETIC RESONANCE IMAGING

MRI currently plays a crucial role in the primary staging of rectal cancer by leading the therapeutic management. MRI shows high accuracy in the assessment of CRM and sphincter invasion assessment<sup>[3,4,41-43]</sup>, and high resolution T2 weighted images are considered the standard sequences to evaluate rectal cancer<sup>[44,45]</sup>.

However, when it comes to restage rectal cancer, MRI utility remains debatable. Several Authors have reported a reduction of its accuracy after neoadjuvant regimen<sup>[46,47]</sup>. Accuracy in predicting rectal wall invasion is 50% (sensitivity, 100%; specificity 35%) and nodal involvement is 65%<sup>[48]</sup>. Prediction of CRM is reported to be 66%-85%<sup>[37,43]</sup>.

The disappointing accuracy of MRI imaging in restaging rectal cancer is due both to overstaging and understaging<sup>[48]</sup>. Typically, overstaging, in the assessment of rectal wall invasion, occurs because after radiotherapy the responding tumors can be replaced by fibrosis, inflammatory and vascular proliferation<sup>[7,48]</sup>. This often results in overstaging T1 or T2 tumors<sup>[46,47]</sup> because tumors are surrounded by diffuse hypointense tissue infiltration<sup>[48]</sup> and the thickness caused by fibrosis is overestimated by MRI. Another common cause of overstaging is radio-induced ulceration or proctitis<sup>[48]</sup>. Understaging is usually due to the inability to detect a small residual tumor overwhelmed

by fibrotic tissue<sup>[46,47]</sup>. To overcome this issue, Kim *et al*<sup>[48]</sup> suggested that comparison of both pre- and post neoadjuvant course should be mandatory to improve the accuracy of MRI restaging. Position, extension, and signal intensity of the tumor are to be considered the key points to compare MRI images before and after neoadjuvant course<sup>[48]</sup>. Measurement of tumor size by three dimensional MR volumetry can be effective to establish tumor downsizing and it has shown good correlation with ypT stage after neoadjuvant regimen<sup>[49,50]</sup>. Perfusion MRI imaging is able to determine tumor vascularization which reflects aggressiveness of the tumor. The microcirculation enhancement could suggest an increased tumor angiogenicity. Thus this technique is reported to be effective in predict tumor response to neoadjuvant course<sup>[51-53]</sup>. Moreover, in diffusion weighted MRI, apparent diffusion coefficient (ADC) could be a useful parameter to predict responsiveness of tumor to neoadjuvant treatment. ADC reduction has been associated to cell apoptosis and increased response to radiotherapy<sup>[54,55]</sup>. It is crucial also to consider that some histological types of adenocarcinoma have different behavior under CRT and different appearance at MRI. For instance, mucinous adenocarcinoma is more aggressive than usual adenocarcinoma and its typical feature is the production of mucin. This histological type is considered poor responder to neoadjuvant and, noteworthy, the great amount of mucin leads to misinterpretation of MRI imaging<sup>[56]</sup> because of its high signal intensity on T2 weighted images<sup>[48,57]</sup>.

Nodal staging by MRI usually relies on size criteria. Typically a lymph node is considered malignant when its short axis measure over 0.5 cm<sup>[58,59]</sup>. It has been reported that also the examination of imaging features such as undefined edges, dissimilar signal enhancement within the node could increase the accuracy of MRI<sup>[39,60]</sup>. Nonetheless, due to fibrosis, undefined borders might be detected after chemoradiotherapy in negative nodes<sup>[48]</sup>. Therefore lymph nodes restaging often results in overstaging because, usually, alteration of nodes structure after radiotherapy is associated with tumor invasion<sup>[46,47]</sup>. New promising strategies using lymph node specific paramagnetic nanoparticles have been reported to increase the accuracy in detecting micro metastasis<sup>[61-63]</sup>.

## PET

Positron emission tomography using <sup>18</sup>F-fluoro-2'-deoxy-D-glucose (FDG-PET) is a diagnostic modality that visualizes the cellular glucose metabolism; it exploits the enhanced glycolysis in tumor cells to distinguish cancer from surrounding tissue with normal metabolic activity. Nowadays functional PET images are coupled with anatomical computed tomography scan so that PET/CT is normally employed for better tumor localization and improvement of diagnostic accuracy<sup>[64,65]</sup>.

PET/CT has been used as noninvasive tool in rectal cancer patients, after neoadjuvant CRT, to detect metabolic activity in the residual tumor and to assess change induced by the treatment<sup>[66,67]</sup>. There is however lack of

uniformly regarding several issues: time interval between end of treatment and examination, parameters used to evaluate tumor response, and criteria to define and measure response.

Radiotherapy and chemotherapy cause tissue inflammation with accumulation of FDG uptake<sup>[68,69]</sup>, since this reaction may last up to several months from the end of treatment, the choice of the time interval to perform the examination is of crucial importance. In addition radiotherapy and chemotherapy can produce a confounding effects called “stunning” a reversible phenomenon characterized by temporarily decrease of glucose metabolisms in viable tumor cells, lasting several weeks. Although the optimal time for the acquisition of PET images has not been established, the control is performed by most authors after 4-6 wk from the end of CRT; it seems that earlier restaging could underestimate tumor response<sup>[70]</sup>.

Different parameters can be used to evaluate tumor response: maximum standardized uptake value (SUV<sub>max</sub>), absolute difference ( $\Delta$ SUV<sub>max</sub>), mean standardized uptake value (SUV<sub>mean</sub>), percent SUV max difference (response index RI), and change in total lesion glycolysis ( $\gamma$ TLG). Depending on the adopted criteria sensitivity and specificity may vary widely. Moreover different cut off value are reported for each parameter producing different diagnostic accuracy. In the majority of the studies the evaluated end point is response to treatment, in relation to regression in T stage or TRG (tumor regression grade). It is important to underline that, for the reasons previously mentioned, and for the limited spatial resolution of PET, that ranges between a 0.4- and 1.0-cm<sup>[71-74]</sup>, it is almost impossible to distinguish major to complete pathological response and therefore to find out  $\gamma$ T0N0 tumors. Sensitivity and specificity of FDG-Pet in predicting response, irrespectively from criteria and cut off value, range between 45%-84.5% and 79%-81%<sup>[73,75-79]</sup>. Few authors evaluated the relation between PET and complete pathological response. In the series of Cho *et al*<sup>[64]</sup> 18F-FDG PET/CT correctly predicted three of the four patients with a pathologic complete response after preoperative CRT. While the only patient with complete response at histopathology was correctly detected by visual FDG-PET analysis by Denecke *et al*<sup>[80]</sup>.

In conclusion although FDG-PET can be considered a promising tool to assess metabolic response after neoadjuvant treatment and to recognize patients more prone to respond to radio chemotherapy from non responders, its role in defining complete response to tailor the therapy is far to be reached.

## CONCLUSION

Neoadjuvant course is effective in producing downstaging and downsizing of locally advanced rectal tumor. Tumor response to such treatment has been significantly associated with improved outcome after surgical resection<sup>[81,82]</sup>.

Enthusiasm about these findings has drove inves-

tigators to sphincter preserving and organ sparing surgery<sup>[83,84]</sup>. In this setting, trans-anal resection of partial responder tumor with negative lymph node assessment by pelvic imaging could be considered as paradigm organ sparing resection.

Moreover the effects of cytoreduction, provided by multimodality treatment, can produce complete clinical response (absence of clinically detectable tumor) or complete pathological response (absence of viable tumor cells at pathology examination after cancer resection) in up to 30% of patients<sup>[5,23,85-87]</sup>. Given that rectal resection is related to significant morbidity, several authors have recommended careful “wait and see strategy” in clinical complete response cases<sup>[13,88-90]</sup>. In this setting rectal cancer restaging after multimodality treatment has been claimed to provide adjustment of the surgical conduct.

Current imaging techniques have been reported to be highly accurate in the primary staging of rectal cancer. On the other hand, radiotherapy and chemotherapy course produce deep modifications on cancer tissue and on surrounding structures. As a result, when used to restage rectal cancer after CRT, the same imaging techniques produce inconstant results. Indeed pathological T stage and lymph nodes status prediction has been shown to be far less inaccurate when compared to primary staging.

Overstaging is a basic issue of current imaging modality. The overstaging is commonly due to the inability of distinguish residual tumor from radio-induced desmoplastic reaction and overgrowth fibrosis in the surrounding tissue. False positive diagnosis can clearly lead to over treat patients that indeed could take advantage of organ sparing surgery such as local excision for  $\gamma$ T1 N0 tumors. Moreover this possibility could be considered for high surgical risk patients thus avoiding morbidity and mortality of rectal resection.

On the contrary, understaging could lead to consider local excision in patients with occult mesorectal positive lymph node, thus producing a non-oncological resection with consequent reduction of survival. Furthermore it has been reported that, after radiotherapy, local recurrence could be more aggressive than native tumor and the situation could be more concerning when leaving untreated patients with complete response without surgically removing the site of the tumor<sup>[91]</sup>.

However, Habr-Gama *et al*<sup>[13]</sup> found no significant difference in terms of survival and disease free rate when comparing patients with complete clinical response undergoing “wait and see policy” and patients with histologically proven complete response after surgery. Interestingly this group, when assessing complete pathological response, mainly relies on direct endoscopic visualization of rectal mucosa and uses additional radiological studies only in case of recurrence suspicion<sup>[24]</sup>.

Although currently available imaging techniques display an overall low accuracy in restaging rectal cancer, CT scan and MRI are efficient in excluding tumor extent to adjacent organs (T4 tumor) and CRM invasion<sup>[57]</sup>.

Clear assessment of lymph node status should be provided when considering local tumor excision due to the risk of leaving positive mesorectal nodes. Prediction of lymph node positivity is reported to be still poor. Moreover there is no consensus about the standard criteria to define lymph nodes positivity. It is clear that the size measurement only is not reliable and analysis of nodal contour, shape and structure has to be considered to improve the accuracy of restaging.

In conclusion modern imaging techniques are unreliable in restaging rectal cancer after CRT given the low correspondence between pathological status prediction and actual pathological assessment. In our opinion imaging evaluation patterns are to be reexamined to reduce the false positive and false negative percentage and to broaden diagnostic accuracy.

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## Probiotics and irritable bowel syndrome

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### Abstract

Irritable bowel syndrome (IBS) is common gastrointestinal problems. It is characterized by abdominal pain or discomfort, and is associated with changes in stool frequency and/or consistency. The etiopathogenesis of IBS may be multifactorial, as is the pathophysiology, which is attributed to alterations in gastrointestinal motility, visceral hypersensitivity, intestinal microbiota, gut epithelium and immune function, dysfunction of the brain-gut axis or certain psychosocial factors. Current therapeutic strategies are often unsatisfactory. There is now increasing evidence linking alterations in the gastrointestinal microbiota and IBS. Probiotics are living organisms which, when ingested in certain numbers, exert health benefits beyond inherent basic nutrition. Probiotics have numerous positive effects in the gastrointestinal tract. Recently, many studies have suggested that probiotics are effective in the treatment of IBS.

The mechanisms of probiotics in IBS are very complex. The purpose of this review is to summarize the evidence and mechanisms for the use of probiotics in the treatment of IBS.

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**Key words:** Probiotics; Irritable bowel syndrome; Visceral hypersensitivity; Brain-gut axis; Immune function

**Core tip:** Irritable bowel syndrome (IBS) is a common gastrointestinal problem and its etiopathogenesis is not fully understood. So the treatments of IBS are based on the predominant symptom. But these treatments are often unsatisfactory. Probiotics have numerous positive effects in the gastrointestinal tract. Many studies have shown that probiotics are effective in the treatment of IBS. In this review, we have summarized the efficacy, the safety and the mechanisms of probiotics in IBS.

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### INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most frequent gastrointestinal problems. IBS has a prevalence of 12%-20% of the population worldwide and 18%-23% in China, and it is 2-3 times more common in women than men<sup>[1]</sup>. About 20%-38% of IBS patients will consult their doctor, accounting for 12% of visits to general practitioners, 28% of visits to specialists, and a significant percentage of visits to outpatient departments of tertiary level hospitals<sup>[2]</sup>.

IBS is characterized by abdominal pain or discomfort, which is relieved by defecation or the passage of gas,

and is associated with changes in stool frequency and/or consistency, without physical, radiological or endoscopic abnormalities or laboratory findings indicating organic disease<sup>[3]</sup>. Abdominal bloating or distension affects 15%-30% of the general population, but is increased to 75%-90% in female IBS patients and constipation-predominant IBS<sup>[4]</sup>. In all, 28% of IBS patients suffer from abdominal bloating all the time, which is slightly less than abdominal pain (33%)<sup>[5,6]</sup>. Many IBS patients state that abdominal discomfort is greater than abdominal pain. It is worse in the afternoon and evening, but better at night; it becomes worse when standing and is relieved when in a supine position. There is no correlation with defecation or retaining flatus, although it is worsened by eating and menstruation. Other gastrointestinal symptoms are also common: incomplete defecation, burning pain on defecation, urgency to defecate, rectal tenesmus, esophageal balloon, heartburn, chest pain, early satiety, abdominal bloating or distension, flatulence; and extragastrointestinal symptoms: asthenia, adynamia, headache, dizziness, sleep disorders, pollakiuria, neck pain, back pain, dysmenorrhea, dyspareunia, fibromyalgia. IBS patients have more psychosocial or mental complaints, and they are absent from work due to acute common diseases. This comorbidity has a significant influence on the patient's clinical and diagnostic management, and therapeutic plan. Although the symptoms of IBS are used to establish the suspected diagnosis, individually they are not sufficiently sensitive or specific. Predictive value increases when patients are aged < 50 years and an absence of warning symptoms or signs are considered, which is not the case with another common functional digestive disorder, functional dyspepsia.

Diagnosis is fundamentally through clinical symptoms, and is based on the Rome III criteria of 2006, with the isolated exclusion of metabolic or organic diseases (benign or malignant) according to the patient's personal or family history. If there is abdominal bloating or distension, it is advisable to rule out lactose, fructose or trehalose intolerance, an excessive intake of insoluble fiber, and small intestinal bacterial overgrowth. According to Rome III there are several types of IBS: (1) diarrhea-predominant IBS; (2) constipation-predominant IBS; (3) alternating IBS (sometimes diarrhea, sometimes constipation); and (4) undefined IBS. In any of these types there may be psychological, psychosocial or psychiatric alterations, or an exaggerated intestinal response to everyday stress. It seriously affects the quality of life of those who suffer from it, and accounts for the use of large amounts of healthcare resources.

IBS patients are known to self-medicate very often. A review of the use of OTC drugs and medicines shows that they take antacids, proton pump inhibitors, fiber and laxatives, antidiarrhea agents, antispasmodics, antidepressants, sedative-hypnotics, and analgesics more frequently<sup>[7]</sup>. Treatment of IBS has been based on the predominant symptom. It must be individualized, emphasizing the absence of a serious or worrying condition,

and straightforwardly explaining the nature and cause of the symptoms. But the therapies available at present (spasmolytics or antidepressants at low doses for the pain, anti-diarrhea agents or 5HT<sub>3</sub> antagonists for diarrhea, lubiprostone, linaclotide, bulk-forming laxatives or 5HT<sub>4</sub> agonists for constipation) have only proven slightly more effective in comparison with placebo, and none have been capable of altering its natural course, as the effect disappears as soon as treatment is stopped<sup>[8-11]</sup>. At the same time IBS patients often resort to alternatives such as medicinal herbs, homeopathy, acupuncture, antibiotics, hypnosis or psychotherapy, but randomized controlled studies with these types of therapy are compromised by their low methodological quality, and in general these treatments are not recommended<sup>[12,13]</sup>.

Now there is a tendency to return to an etiopathogenesis-based or pathophysiology-based therapeutic approach, attempting to influence the possible existence of intestinal dysbacteriosis, altered intestinal fermentation and intestinal microbiota, excess production or alteration in the management of intestinal gas, but also subclinical mucosal inflammation, especially in patients in whom the onset of IBS followed an episode of acute bacterial gastroenteritis, antibiotics or immunosuppressants, because these can affect the normal balance of intestinal microflora<sup>[14,15]</sup>. Thus, one therapeutic approach in IBS patients could be to modulate intestinal microflora to correct an imbalance such as probiotics.

## MECHANISMS OF IBS

The etiopathogenesis, which is not fully understood, may be multifactorial, as is the pathophysiology, which is attributed to alterations in gastrointestinal motility, visceral hypersensitivity, intestinal microbiota, gut epithelium and immune function, dysfunction of the brain-gut axis or certain psychosocial factors. Some studies have demonstrated that there may be some autonomic dysfunction with an increased or sustained response to normal psychophysical stress in IBS patients; and further studies have found that salivary chromogranin, a derivative of catecholamines that is released in response to acute stress, is high in IBS patients and may be reduced using muscle relaxation techniques<sup>[16]</sup>. At the same time the management of intestinal gas is different in IBS patients and control subjects; whilst the latter expel the gas infused into the jejunum rapidly, the former retain it, causing them symptoms, regardless of whether or not intestinal gas production is increased<sup>[17]</sup>. Although genetic factors play a minor role compared to learned behavior, they do influence symptomatic expression and especially therapeutic response<sup>[18,19]</sup>. The specific mechanisms have been reported as follows.

### Visceral hypersensitivity

There is visceral hypersensitivity in IBS patients, expressed by: (1) increased ileocolonic response to bile acid perfusion; (2) reduced rectal adaptation to distension; and

(3) reduced pain threshold to rectal distension. Hypervigilance to digestive events would contribute to visceral hypersensitivity, perhaps due to inadequate processing of afferent information in certain areas of the central nervous system, such as the anterior cingulate cortex, the amygdala, and the dorsomedial frontal cortex, as shown by dynamic magnetic resonance imaging studies, which indicate an increase in the vascularization of these areas in response to colon stimulation that does not occur in controls<sup>[20,21]</sup>. IBS patients are more prone to take surgical interventions such as appendectomy, cholecystectomy and hysterectomy to relieve symptoms; this is because these symptoms can be mistaken for other diseases. Now it was pointed out that patients who have undergone cholecystectomy with follow-up as a cohort for 10-15 years have twice as high a risk of suffering from IBS<sup>[22]</sup>. It is assumed that the continued presence of bile salts in the colon may give rise to symptoms of IBS, as these patients are known to have a hyper response to these substances.

### Immune activation

Despite being considered a non-organic disease, it has certain organic components problems, especially in post-infectious IBS (PI-IBS)<sup>[23]</sup>. Although PI-IBS explains only 23%-35% of IBS cases, it shows the relationship between exposure to the infectious agent → mucosal and systemic inflammation → clinical expression of IBS. Increased levels of peripheral cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, IL-6, intraepithelial lymphocytes and CD3 and CD25 cells in the lamina propria, were found in IBS patients<sup>[24,25]</sup>. At the same time, a nonspecific microscopic inflammation with increased mast cells and neutrophils were found in mucosal biopsies. There is speculation that IBS symptoms may result from cytokine or mediator secretion by these inflammatory cells. Increased production of proinflammatory and decreased production of anti-inflammatory cytokines was in fact demonstrated<sup>[26]</sup>. In addition, a low-grade inflammatory infiltration and activation of mast cells in proximity to nerves in the colonic mucosa were found and may be involved in the pathogenesis of pain episodes. These results suggested that immune activation plays an important role in IBS patients.

### Intestinal hormone secretion

Although little is known about intestinal hormone secretion in IBS patients, high levels of cholecystokinin and vasoactive intestinal polypeptide were found in the plasma and rectosigmoid mucosa, whilst substance P (SP) and somatostatin were normal<sup>[27]</sup>. However, neuropeptide Y levels in the plasma and rectosigmoid mucosa are lower in diarrhea-predominant IBS, but not in constipation-predominant IBS.

### Intestinal microflora

Host intestinal microflora interactions lead to immune tolerance, sustain the function and integrity of the epithelial barrier and its vascularization, promote the devel-

opment and maintenance of gut-associated lymphoid tissue (GALT), and are essential for the development of jeuno-colonic motility under physiological conditions. Some studies have found that a dysregulated interaction between the intestinal bacteria, the gut barrier, the intestinal immune system and GALT play a fundamental role in IBS. Patients have a different composition of intestinal bacteria such as *Bacteroides* spp., *Bifidobacterium* spp., *Lactobacilli* spp. and others compared to healthy controls. When ribosomal RNA-based microbiological technologies were used with cloning and sequencing of genes, these differences were even more significant. These new views in the pathogenesis of IBS changed the treatment approach to influence the composition of the gut microbiota and to stop the inflammatory process and to interfere in the dysregulated intestinal immune system. Among several treatment options, the use of probiotics seemed to be promising.

## PROBIOTICS

Since Elie Metchnikoff first published “The prolongation of life: optimistic studies” in 1907, the concept of probiotics is 100-year-old now. In 1998 Guarner *et al* defined probiotics as living microorganisms that have benefits for the gastrointestinal tract and its immune function after being ingested. In 2003 the concept of “immune function of probiotics” was introduced, including the fact that probiotics modulate the immune response throughout the mucosa associated lymphoid tissue system; this idea maintains the concept that intestinal mucosa and intestinal microflora constitute an anatomical-functional unit that regulates both the cell-mediated and humoral immune responses and the local production of cytokines. In 2008, a review defined probiotics as living microorganisms, which when ingested in certain numbers, exerted health benefits beyond inherent basic nutrition.

Now probiotics are becoming an increasingly important part in the diet of everyday life, as their general and gastrointestinal beneficial effects are being gradually proven. It has become necessary to harmonize marketing criteria, evaluate the efficacy of probiotics, and correctly define what is a probiotic, what the effective doses are, and whether they are completely safe. Probiotics are defined at three levels: genus, species and strain; it is essential to understand that their properties depend on all three and cannot be assigned to other similar ones, even if they share the same genus and species. The therapeutic benefits of a strain of probiotics cannot be extended to other strains, and their efficacy has to have been proven individually. Therefore, we shall now examine evidence available to date. Usually probiotics are certain types of Streptococcus, Lactobacilli, and Bifidobacteria, but also other non-pathogenic bacilli such as *E. coli*-Nis1917 and yeasts such as *Saccharomyces boulardii*. The best known and most widely used probiotics are *Lactobacillus plantarum* 299v, *Lactobacillus rhamnosus* LGG, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium*

*infantis*, *lactis* or *brevis*. Probiotics can be administered not only as functional foods, but also in pharmaceutical forms similar to medicines. For a probiotic to be effective, five conditions must be fulfilled: (1) it must not be toxic or pathogenic; (2) it must have a proven beneficial effect on the host; (3) it must contain a sufficiently large number of viable microorganisms per unit; (4) it must be capable of surviving in the intestine, reproducing, maintaining itself, and having intraluminal metabolic activity; and (5) it must remain viable during storage and use.

## EFFICACY AND SAFETY OF PROBIOTICS IN IBS

The evidence about probiotics in the treatment of IBS is type II (grade B). It is because not all probiotics have been shown to be equally effective, and there are still relatively few studies, some of which are not randomized controlled trials (RCTs), some with a single probiotic, others with multiple species, and even some combined with prebiotics. The Clinical Practice Guideline about IBS in the Treatment section concluded that probiotics could improve the global symptoms of IBS patients based on some RCTs. In recent years there have been numerous publications, which have confirmed the effect<sup>[28-32]</sup>. Many good clinical studies using mainly lactobacilli and bifidobacteria alone or in combination have been published<sup>[33-37]</sup>. In general, it appears that probiotics are effective in IBS patients. However, looking at single trials, it seems that probiotics are more effective on single symptoms than on the entire IBS. It is difficult to compare because of variations in the study design, probiotics strains used, doses administered and formulation. There is still a need for further studies to determine the most effective species and strain, the right doses and to clarify whether a combination is better than a single strain.

The safe use of probiotics is an absolutely crucial matter. Conventional toxicology and safety evaluation has limitations for the safety assessment of probiotics. Vigorous debate continues on what constitutes appropriate safety testing for novel probiotic strains proposed for human use. In recent years several organizations have formulated approaches to assess the safety of probiotics. The Joint FAO/WHO working group on drafting guidelines for the Evaluation of Probiotics in Food proposed a framework consisting of strain identification and functional characterization, followed by safety assessment and Phase 1, 2 and 3 human trials. These studies have shown that the use of probiotics in IBS patients and healthy subjects involves a very low risk of bacterial complications, although over 80 cases of bacteremia have been reported in Finland, associated with severe prior comorbidities or surgery, and always with *Lactobacillus*<sup>[38]</sup>. Lactobacilli, Bifidobacteria and other commensal microorganisms are generally regarded as safe, although certain doubts have been raised regarding their use at massive doses in immunodepressed patients or in those who undergo intestinal resection due to benign or malignant disease<sup>[39]</sup>. Other

microorganisms such as *Enterococcus* may be opportunistic pathogens depending on host conditions.

In conclusion, the concept of using probiotics in IBS patients is interesting and it appears that certain probiotics strains are efficacious on several symptoms and very safe.

## MECHANISMS OF PROBIOTICS IN IBS

It is well known that probiotic strains have numerous positive effects in the gastrointestinal tract. The beneficial effects of probiotics in IBS could be explained by increasing the mass of beneficial bacteria in the digestive tract, decreasing bacterial overgrowth in the small bowel and reversing the imbalance between the pro- and anti-inflammatory cytokines. Probiotics can also reinforce the intestinal mucosal barrier and normalize the motility of the digestive tract and its visceral sensitivity. Recently it was also demonstrated that some lactobacilli strains may modulate intestinal pain attacks by inducing the expression of  $\mu$ -opioid and cannabinoid receptors in the intestinal epithelial cells. In conclusion, probiotics have various actions: (1) physical barrier effect; (2) competition for nutrients; (3) metabolic interactions; (4) bacteriocin production; (5) reinforcement of the intestinal mucosal barrier; (6) reduction of intestinal permeability and bacterial translocation; and (7) regulation of the intestinal inflammatory response by modulating the secretion of cytokines and the immune response. The specific mechanisms of probiotics have been reported as follows.

### *Inhibition of pathogen binding*

Bacteria in the gut compete for nutrients and space. Probiotics have been demonstrated to adhere to a range of human intestinal cell lines and commonly found in mucosal biopsies of healthy individuals<sup>[40]</sup>. Probiotics reduce the adherence of pathogenic bacteria on the epithelial cells and thus the ability of pathogenic bacterial translocations. Probiotics can control the growth of pathogenic bacteria and regulate intraluminal fermentation by stimulating the secretion of bacteriocins and defensins, modulate signal transduction (*e.g.*, NF- $\kappa$ B) and influence the innate/adaptive immune system (*e.g.*, IgA secretion).

### *Enhanced barrier function*

Probiotics have been demonstrated to enhance barrier function. The probiotic VSL#3 can protect cultured T84 monolayers from invasion by *Salmonella* by enhancing barrier function<sup>[41,42]</sup>. Our studies have also found that VSL#3 protected the epithelial barrier and increased the tight junction protein expression in vivo and in vitro by activating the p38 and extracellular regulated protein kinases (ERK) signaling pathways<sup>[43]</sup>.

### *Immune function*

Some studies have demonstrated that probiotics have an anti-inflammatory effect. In a study from Cork, assessing individual bacterial species, improvement in symptoms

with Bifidobacteria was associated with changes in the relative production of anti-inflammatory (IL-10) to pro-inflammatory (IL-12) cytokines<sup>[44]</sup>. Animal model and human studies have evaluated immunologic modulation with specific probiotics. The potential anti-inflammatory effect of *Lactobacillus reuteri* in an experimental rodent study demonstrated an inhibition of TNF- $\alpha$ -induced production of IL-8<sup>[45]</sup>. *Lactobacillus casei* can also significantly decrease TNF- $\alpha$  release in ileal tissues from an experimental rodent study<sup>[46]</sup>. Other studies also evaluated the anti-inflammatory effects of probiotics and demonstrated activity against cytokines, including interferons.

The main action on the immune function of probiotics is carried out by dendritic cells (DCs), antigen presenting cells for T lymphocytes that are found in mucosae, lymphoid tissues, lymph, lymph nodes, spleen and peripheral blood. DC phenotype and cytokine production is modulated by the intestinal microflora; furthermore, these cells are involved in the local immune response to B lymphocyte activation and IgA synthesis by plasmacytes.

### Colonic transit and motility

Effects of probiotics on colonic transit have been reported in IBS patients with predominant bloating. In IBS patients with predominant bloating, the colonic transit was significantly retarded with probiotic VSL#3 relative to placebo. The effect on transit was not associated with the worsening of bowel function. Bazzocchi *et al* showed that the colon's reflex motor responses to balloon distension were reduced during an open-label study with VSL#3. Further studies are indicated to explore the mechanism of the retarded transit of stools and potential effects on colonic sensation and fermentation of nutrients reaching the colon.

### Effect on intraluminal milieu

Effects of probiotics on intraluminal milieu have been reported as follows: (1) reduction of intracolonic gas of bacterial origin due to an increase in Lactobacilli and Bifidobacteria, and a consequent decrease in the proportion of Clostridia and Veillonella; (2) normally metabolize nutrient substrates reaching the colon with the formation of gas and increase in the production of intracolonic short chain fatty acids (SCFA) and a consequent improvement in colonic propulsion. The SCFA may induce propulsive contractions and accelerate transit or enhance fluid and sodium absorption in the colon<sup>[47]</sup>. Thus, alteration in the resident colonic flora with administration of probiotics may modify the colonic metabolism of nutrient substrates to alter colonic transit and fluid fluxes; and (3) reduced malabsorption of bile acids in diarrhea predominant IBS, because Lactobacilli and Bifidobacteria are capable of deconjugation and absorbing bile acids, thus reducing luminal bile salt load to the colon, which reduces colonic secretion and mucosal permeability changes.

### Alterations in visceral hypersensitivity

Some studies have shown that *E. coli* Nissle 1917 inhibits

the visceral hypersensitivity associated with trinitrobenzene sulphonic acid (TNBS) colitis and *L. paracasei* inhibits the visceral hypersensitivity associated with inflammation in healthy mice in whom the bacterial microbiota have been disturbed by antibiotics<sup>[48]</sup>. This latter study showed a clear anti-inflammatory effect and also an inhibition of SP staining, a marker of afferent pain pathways, which was increased after the antibiotic treatment<sup>[49]</sup>. This effect on neuropeptides is of particular interest given the key role visceral hypersensitivity is believed to play in IBS and the recent demonstration of increased SP and transient receptor potential vanilloid 1 receptor (TRPV1) positive fibres in IBS mucosal biopsies<sup>[50]</sup>. Probiotics can regulate the action of mastocytes in the vicinity of nerve endings within the intestinal lamina propria. An entirely novel mode of action of probiotics has recently been demonstrated in which *L. acidophilus* increased the expression of l-opioid and cannabinoid receptors in normal animals, a phenomenon which was associated with an inhibition of visceral sensitivity equivalent to that of morphine 0.1 mg/kg<sup>[51]</sup>.

## FECAL MICROBIOTA TRANSPLANTATION AND IBS

Fecal microbiota transplantation (FMT), or infusion of a fecal suspension from a healthy individual into the gastrointestinal tract of another person to cure a specific disease, is best known as a treatment for recurrent *Clostridium difficile* infection. The earliest and most frequently quoted report of FMT is that by Eiseman *et al*<sup>[52]</sup>, who successfully treated patients using fecal enemas in the 1950s.

FMT also has been used successfully for IBS. Recent studies have shown that the intestinal microbiota play an important role in immunity and energy metabolism and that an imbalance in our commensal intestinal bacteria can predispose to disease development<sup>[53-56]</sup>. Re-establishment of the wide diversity of intestinal microbiota *via* infusion of donor feces into the colon is the proposed mechanism by which FMT results in clinical improvement in patients with IBS.

FMT is by no means a new therapeutic modality, however, it did not receive public attention until recently, after several studies were published showing that stool is a biologically active, complex mixture of living organisms with great therapeutic potential for *Clostridium difficile* infection<sup>[57-59]</sup> and other gastrointestinal diseases<sup>[60-63]</sup>.

Unlike the concept of probiotics, which alter the metabolic or immunological activity of the native gut microbiota, the premise of FMT has always been to introduce a complete, stable community of gut microorganisms, which are aimed at repairing or replacing the disrupted native microbiota. Engraftment of donor microbiota was accompanied by normalization of the patient's bowel function. In addition, other mechanisms could be involved that might explain how FMT works. The metabolic activities of gut bacterial species can have

consequences both locally, on the gut mucosa, and systemically. Disruption of these bacterial species can result in potentially harmful metabolic alterations, leading to the partitioning of toxic substances across the gastrointestinal mucosa where these substances are absorbed into systemic circulation. Gustafsson *et al*<sup>[64]</sup> analyzed gut microbiota metabolism pre-FMT and post-FMT in patients with antibiotic-associated diarrhea and found marked disturbances in the majority of microflora-associated characteristics in patients with antibiotic-associated diarrhea. Administration of a human fecal enema corrected these alterations and relieved diarrhea.

## CONCLUSION

Probiotics are used regularly in most populations regardless of medical indications. There is reasonable evidence of a modest benefit in IBS patients. Selection of those who will respond requires a better understanding of exactly what the mode of action is in IBS. Like most therapies in IBS probiotics are unlikely to be beneficial for all patients. However, given their impressive safety profile, a trial of probiotics is certainly worth considering. Care must be taken to recommend the exact strain or species that has shown benefit in treating IBS, and not to extrapolate success of one probiotic species to another. In addition, further large good quality trials are needed to predict which patient groups are most likely to respond to probiotics, perhaps through fecal microbial profiling.

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## Tolerance and chimerism and allogeneic bone marrow/stem cell transplantation in liver transplantation

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### Abstract

The liver has particular tolerogenic properties that allow its spontaneous acceptance in some animal species. Liver structure is considered to favor a tolerogenic environment. The peripheral tolerance mechanisms also play a role in spontaneous tolerance to liver graft. In a clinical setting, the main challenge nowadays facing liver transplantation is minimization of immunosuppression with the goal of donor-specific tolerance. Mechanisms involved in tolerance to transplanted organs are complex and partly unknown. A significant mechanism in tolerance induction is chimerism. Chimerism can be induced through transplantation of allogeneic donor bone marrow/stem cells under appropriate host conditioning. This review focuses on the tolerance mechanisms in liver transplantation and highlights the role of chimerism and allogeneic bone marrow/stem cell transplantation in tolerance development.

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**Key words:** Immunotolerance; Chimerism; Bone marrow transplantation; Stem cell transplantation; Liver transplantation

**Core tip:** The liver is considered an immune privileged organ. The main challenge facing liver transplantation

is to induce donor-specific tolerance. Numerous reports have documented the phenomenon of microchimerism in liver transplant recipients. Most have demonstrated that higher levels of chimerism in liver transplantation are associated with reduced incidence of acute rejection and better initial graft acceptance. Mechanisms involved in chimerism-induced tolerance have only been partly elucidated. Chimerism can be induced through transplantation of allogeneic donor bone marrow cells under appropriate host conditioning and represents a clinically feasible approach for the induction of durable liver transplantation tolerance.

Wu SL, Pan CE. Tolerance and chimerism and allogeneic bone marrow/stem cell transplantation in liver transplantation. *World J Gastroenterol* 2013; 19(36): 5981-5987 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/5981.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.5981>

### INTRODUCTION

The availability of non-specific immunosuppressive agents has allowed liver transplantation to become an established treatment of end-stage liver disease. With the introduction of new immunosuppressants the incidence of acute rejection has considerably decreased, and transplanted patient survival is now 83% and 70% after 1 and 5 years, respectively<sup>[1]</sup>. However, immunosuppressive drugs broadly suppress the immune system, and their use is associated with an increased risk of neoplasms, opportunistic infections, and end-organ toxicity<sup>[2,3]</sup>. Thus, the main challenge nowadays facing liver transplantation is minimization of immunosuppression with the goal of donor-specific tolerance. Tolerance is defined as a state of donor-specific hyporesponsiveness in the recipient in the absence of immunosuppression<sup>[4]</sup>. Mechanisms involved in tolerance to transplanted organs are complex and partly unknown. This review focuses on the tolerance mechanisms in liver transplantation and to highlight

the role of chimerism and allogeneic bone marrow/stem cell transplantation in tolerance development.

## LIVER AS A PRIVILEGED IMMUNE ORGAN

It is generally recognized that after clinical liver transplantation, the incidence of chronic rejection is lower than after transplantation of other organ grafts. Among animal species such as pigs, and selected rat and mouse combinations, liver transplants may be performed with no immunosuppression<sup>[5-7]</sup>. In selected patients with liver transplantation, immunosuppressive therapy can be withdrawn without occurrence of graft rejection<sup>[8-10]</sup>. Furthermore, co-transplantation of a liver allograft can prevent rejection of other organ grafts from the same donor<sup>[11,12]</sup>. The transplanted liver may even behave as an immunosuppressor when transplanted to animals suffering rejection after pancreas transplantation<sup>[13]</sup>. Liver allografting also reverses ongoing rejection of the heart<sup>[14]</sup>. All of these facts had led people to consider the liver to be an immunologically privileged organ that may be tolerated with less immunosuppression after transplantation, and immunosuppressants can sometimes even be completely withdrawn<sup>[15]</sup>.

## TOLERANCE MECHANISMS IN LIVER TRANSPLANTATION

The mechanisms responsible for this relative tolerogenicity of the liver have only been partially elucidated, and liver structure is considered to have major implications for hepatic immune function. The liver is a place where gastrointestinal tract antigens and alloantigens in transplanted liver are presented to lymphocytes through a complex network of sinusoidal cells and antigen presenting cells (APCs)<sup>[16]</sup>. The context in which liver-resident antigens are presented to T cells favors a tolerogenic environment. Liver sinusoidal endothelial cells (LSECs) have a unique phenotype expressing myeloid cell markers (CD1, CD4 and CD11c), similar to immature dendritic cells (DCs). LSEC-activated CD4<sup>+</sup> lymphocytes cannot differentiate into T helper (Th)1 [interleukin (IL)-2-producing] cells, and express high immunosuppressive IL-10 levels<sup>[17]</sup>. Besides, LSEC-stimulated CD8<sup>+</sup> lymphocytes cannot respond to new antigenic stimuli<sup>[18]</sup>.

A recent study demonstrated that the liver capacity to induce tolerance partly results from *in situ* T-cell activation. It is well known that hepatocytes, as non-professional APCs, may play key roles in regulating immune responses and facilitating tolerance induction<sup>[19]</sup>. Warren *et al.*<sup>[20]</sup> showed intrahepatic lymphocytes and circulating naïve CD8<sup>+</sup> cells could interact with hepatocytes by means of cytoplasmic extensions capable of going through LSEC fenestrations. This local activation of T cells by hepatocytes provides the latter with a significant role as APCs and induces tolerance development in the liver<sup>[21]</sup>.

A number of observations indicate that a high per-

centage of non-conventional lymphocytes-which only rarely are found in peripheral blood-in the liver graft may also play a relevant role in the induction of tolerance<sup>[22]</sup>. Natural killer (NK) cells represent up to 30%-40% of liver lymphocytes, but only 10%-15% of peripheral mononuclear cells. Liver NK cells contain perforin and granzymes, exert stronger cytotoxicity against K562 target cells when compared with blood NK cells, and secrete interferon (IFN)- $\gamma$ , but no IL-10 or Th2 cytokines, upon stimulation with monokines. Liver NK cells seem to emit negative signals to the host T cells upon their migration into the liver following liver transplantation, thus contributing to liver graft tolerance<sup>[23]</sup>.

The peripheral tolerance mechanisms also play a role in liver graft spontaneous tolerance. Bishop *et al.*<sup>[24]</sup> demonstrated after liver transplantation that a large number of donor leukocytes rapidly migrate to secondary lymphoid organs and stimulate production of IL-2 and IFN- $\gamma$  by host CD4<sup>+</sup> cells, which then become depleted and subsequently undergo apoptosis.

A distinct subset of T cells that play an important role in peripheral regulation is natural T regulatory cells (Tregs). They constitutively express CD25, and constitute 5%-10% of peripheral CD4<sup>+</sup> T cells in healthy mice and humans. The CD4<sup>+</sup>/CD25<sup>high</sup> Tregs are best recognized by expression of the transcriptional regulator forkhead box (Fox)P3<sup>[25]</sup>. Dieckmann *et al.*<sup>[26]</sup> showed that human peripheral blood CD4<sup>+</sup>CD25<sup>+</sup> Tregs can suppress allogeneic responses through soluble mediators such as IL-10 and/or transforming growth factor- $\beta$ . It was recently found that peripheral blood CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells increased in patients with liver grafts and might contribute to spontaneous tolerance<sup>[27]</sup>.

Compared with peripheral tolerance, central tolerance phenomena occur primarily in the thymus. So far, two experimental approaches exist in murine models that may generate highly stable central tolerance to allogeneic thymus transplantation or bone marrow transplantation (BMT). Induction of mixed chimerism through transplantation of allogeneic donor bone marrow/stem cells under appropriate host conditioning, is one of the most reliable strategies to induce transplantation tolerance and has been used in clinical practice<sup>[28]</sup>. Next, we focus on the mechanisms and applications of chimerism induced by allogeneic bone marrow/stem cell transplantation in tolerance introduction in liver transplantation.

## CHIMERISM

Chimerism can be defined as a phenomenon in which cells from one individual are present in another individual. Two types of chimerism have been described: microchimerism and macrochimerism. Microchimerism usually occurs when bone marrow/stem cells are transplanted in a conditioned recipient and the donor pluripotent hematopoietic stem cells (HSCs) engraft in the recipient and produce all its lineages, including the donor immune system. Microchimerism arises as a result of migration of passenger leukocytes from a transplanted allograft into an

unconditioned recipient and donor pluripotent HSCs do not engraft, but alternatively hematopoietic-derived cells from the donor organ are produced and migrate systemically. Consequently, not all stem-cell-derived lineages are produced and low levels of donor cells are found in the recipient's blood<sup>[29]</sup>.

## CHIMERISM AND TOLERANCE IN LIVER TRANSPLANTATION

Tolerance induction with live donor leukocytes became the bedrock of modern transplantation after its first description in mice<sup>[30]</sup>. Numerous reports have documented that mononuclear cells of donor type migrate out of the graft after transplantation of solid organs and these cells can persist in the recipient over several years, thus leading to allogeneic microchimerism<sup>[31]</sup>. Jonsson *et al*<sup>[32]</sup> prospectively investigated the peak levels and kinetics of donor leukocyte chimerism in human recipients following liver transplantation. The peak levels of chimerism were observed within the first 48 h following transplantation and ranged from 0.15% to 20% of total peripheral blood mononuclear cells. In almost all patients, there was an early peak level of chimerism that declined over time such that donor leukocytes were only intermittently detectable after 3-4 wk. In another study, Verdonk *et al*<sup>[33]</sup> prospectively collected blood samples of 21 liver transplant recipients up to 3 mo after transplantation. They found donor chimerism in 71% of their liver transplant recipients and chimerism was most frequently found in the first month after transplantation. However, by polymerase chain reaction (PCR) for donor type DNA sequences, microchimerism has been described to be present in patients > 10 years after liver transplantation. Some of these patients were off immunosuppressive treatment for various periods of time and all had good graft functions<sup>[34]</sup>. Stable levels of donor chimerism, in the absence of other major clinical events, may be a marker of transplantation tolerance, and may help to tailor immunosuppressive treatment in liver transplantation<sup>[35]</sup>. As low as 1% donor chimerism is sufficient to induce robust tolerance to donor-specific organs, cells, and tissues<sup>[36]</sup>.

So far, the mechanisms of induction of tolerance by microchimerism are still a matter of speculation. It has been suggested that donor leukocytes of bone marrow origin present in organ grafts represent a functional part of the donor immune system that is incorporated into the recipient's immune system, and a new hybrid immune system is thus established in the recipient and reciprocal bidirectional donor:host tolerance results<sup>[37]</sup>. Starzl *et al*<sup>[38]</sup> make a strong argument that allogeneic microchimerism, in which the donor passenger leukocytes migrate widely into the recipient's lymphoid tissues, is essential for the maintenance of clonal exhaustion-deletion that is induced by the initial flood of passenger leukocytes during the first few weeks after transplantation, and the survival of passenger leukocytes is associated with long-term acceptance of the graft. It is not known what determines the release of donor leukocytes from the graft. These

cells may be stimulated to migrate from the allograft by high local concentrations of tumor necrosis factor- $\alpha$  and IL-1 secreted early after transplantation. Alternatively, they may be mobilized or released by the organ procurement and reperfusion process. Cold preservation of liver allografts may result in injury to adhesion molecules of sinusoidal-lining cells, with the resultant sloughing of viable cells into the sinusoidal lumen<sup>[39]</sup>. Once donor stem cells have engrafted, they coexist and develop together with those of recipient origin giving rise to all hematopoietic cell types. Consequently, not only self-reactive but also donor-reactive thymocytes are intrathymic ally eliminated through negative selection, leading to a robust state of tolerance. Billingham *et al*<sup>[30]</sup> have shown that the induced tolerance is T cell mediated, and thymectomy of recipient mice before establishment of mixed chimerism results in failure to induce tolerance.

According to the concept of the two-way paradigm proposed by Starzl *et al*<sup>[40]</sup>, clinical organ transplantation under immunosuppression involves a double-immune reaction that has host-versus-graft as well as graft-versus-host arms<sup>[41]</sup>. After transplantation, except for the migration of donor hematopoietic cells into host tissues, protecting the allograft, the host's own hematopoietic cells also repopulate the allograft, protecting it from autologous alloreactive T cells<sup>[42]</sup>. Chimeric cells can either be circulating or they can be integrated into the parenchyma, which was first described in transplanted organs in 1969<sup>[43]</sup>. Okabayashi *et al*<sup>[44]</sup> termed it reverse chimerism. In their model, reduced-size livers of the DA rat strain were transplanted into the allogeneic green fluorescence protein (GFP) + Lewis recipients. In this strain combination, a combination of tacrolimus and plerixafor led to indefinite graft survival. Histological examination of the grafts showed a surprisingly high percentage of the liver parenchyma expressing GFP. This unexpected finding suggests that the host's HSCs were mobilized, repopulating the liver and converting to hepatocytes. In other studies, chimeric endothelium and duct epithelium were also found in transplanted livers<sup>[45,46]</sup>. Chimerism was also reported in other transplanted organs: recipient-derived endothelial cells were found in kidney grafts<sup>[47]</sup>; chimeric cardiomyocytes and smooth muscle cells were found in transplanted hearts<sup>[48]</sup>; and chimeric bronchial epithelium and type II pneumocytes were found in transplanted lungs<sup>[49]</sup>. These results are of interest because of their potential clinical application in organ transplantation. It may be conceivable to perfuse a deceased donor allograft with recipient stem cells pretransplantation, thus reversing chimerism and inducing transplantation tolerance.

## ALLOGENEIC BONE MARROW/STEM CELL TRANSPLANTATION IN TOLERANCE INDUCTION IN LIVER TRANSPLANTATION

Many studies suggest that chimerism is essential for tolerance induction in transplantation, therefore, the logical

next step is to augment it. Chimerism can be induced through transplantation of allogeneic donor bone marrow/stem cells under appropriate host conditioning. To surmount physiological and immunological barriers for successful bone marrow cell (BMC) engraftment, various myeloablative or nonmyeloablative conditioning protocols have been developed involving the global elimination of recipient T cells<sup>[50]</sup>. Myeloablative irradiation leads to complete destruction of the hematopoietic repertoire of the host, which is then reconstituted by donor BMCs. Apart from the toxicity of this approach, full donor chimerism is associated with a higher incidence and severity of graft-versus-host disease and some degree of immunoincompetence for primary immune responses, and is therefore clinically undesirable<sup>[51]</sup>. Mixed chimerism can be achieved by nonmyeloablative doses of total body irradiation (TBI) combined with different medication protocols to overcome pre-existing alloreactive T cells in the periphery. Rahhal *et al.*<sup>[52]</sup> prepared mixed chimeras by transplanting  $10^8$  T-cell-depleted allogeneic BMCs into Wistar Furth rats recipients conditioned with 300-600 cGy TBI. An 11-d course of tacrolimus and one dose of antilymphocyte serum were administered postoperatively. Mixed chimerism was initially achieved in almost all recipients and induced long-term acceptance of composite tissue allotransplants. Co-stimulation blockade, consisting of an anti-CD154 monoclonal antibody and the fusion protein cytotoxic T-lymphocyte antigen (CTLA)4-Ig inhibits CD40-CD40L and CD28-CD80/86 interactions between T cells and (allo)antigen-presenting cells<sup>[53]</sup>. Pree *et al.*<sup>[28]</sup> outlined a nonmyeloablative murine BMT protocol including 3 Gy TBI on day 1, a conventional dose of fully mismatched BMCs on day 0, plus a single dose injection of anti-CD154 on day 0 and administration of CTLA4-Ig on day 2. With this protocol, high levels of mixed chimerism (20%-90%) in all tested lineages is induced and maintained for the length of follow-up, and donor skin is accepted permanently in the majority of chimeras, whereas third party skin is rejected promptly.

In humans, BMT-induced mixed chimerism has been shown to confer acceptance of donor-specific skin<sup>[54]</sup> and kidney allografts<sup>[55]</sup> without long-term immunosuppression. Similar results were also observed in the field of liver transplantation. In a pilot study in 1997, donor peripheral blood stem cell (DPBSC) infusions were performed in three recipients of living-related liver transplantation (LRLT). The results, at 20 wk post-transplant, suggested that the levels of donor cells detected in LRLT recipients treated with DPBSC infusions may be higher than that in recipients of cadaver donor liver allografts, indicating that administration of DPBSCs to recipients of liver transplants is a feasible procedure<sup>[56]</sup>. Since then, many human trials of HSC infusion before or after liver transplantation have been performed and tolerance induction observed in the patients<sup>[57-59]</sup>. Tryphonopoulos *et al.*<sup>[60]</sup> perioperatively administered to liver transplant recipients unmodified cadaveric donor bone marrow infusions (DBMIs) ( $5 \times 10^8$ /kg) in order to enhance chimerism. They

found that patients who had DBMI tolerated withdrawal of tacrolimus or cyclosporine-based immunosuppression more often and had 5-6-fold more chimerism. Donckier *et al.*<sup>[61]</sup> reported three patients prospectively enrolled in an original protocol designed to promote graft acceptance in living donor liver transplantation. The protocol relies on the use of donor stem cells administered after liver transplantation as tolerogenic/suppressive cells<sup>[62]</sup>. Post-transplant immunosuppression and conditioning included steroids, rapamycin and antithymocyte globulin. Donor CD34<sup>+</sup> stem cells were infused 7 d post-transplant. The clinical observations demonstrated that donor stem cell infusion combined with recipient conditioning may allow early immunosuppression withdrawal or minimization after liver transplantation.

All of these studies provide hope for liver transplant recipients to be off drugs for the rest of their lives. However, researchers have cautioned that only the healthiest patients will be able to withstand the conditioning regimens that allow donor stem cells to engraft, and for the comprehensive application of bone marrow/stem cell transplantation in solid organ transplantation, there is still a long way to go<sup>[63-66]</sup>.

Establishment of chimerism in donor liver with recipient-type BMCs prior to liver transplantation is another strategy to induce tolerance to the liver graft. Sanada *et al.*<sup>[14]</sup> established chimerism in rat donor liver by intraportal injection of recipient-type BMCs, followed by intramuscular administration of FK506 for 5 d. At 1-2 mo later, livers were harvested and transplanted. No immunosuppressants were used. They found that with livers from rats pretreated with recipient-type BMCs, survival of liver allografts was significantly extended. However, the significance of reverse microchimerism in liver transplantation is still controversial. In a recent study, Aini *et al.*<sup>[67]</sup> compared the proportions of recipient-derived hepatocytes in long-term stable liver allografts and late dysfunctional allografts caused by chronic rejection. They found that hepatocyte chimerism was a constant event. However, the extent of engraftment of recipient-derived hepatocytes does not seem to correlate with the degree of hepatic injury in long-term liver allografts. More importantly, this protocol cannot itself be applicable to clinical allotransplantation because it needs donor preparation long before liver transplantation.

## SUMMARY AND CONCLUSION

The liver has particular tolerogenic properties that allow its being spontaneously accepted in some animal species. Liver structure is considered to favor a tolerogenic environment. The peripheral tolerance mechanisms also play a role in liver graft spontaneous tolerance. A most significant mechanism in tolerance induction is chimerism. The mechanisms of induction of tolerance by microchimerism are still a matter of speculation. Chimerism can be induced through transplantation of allogeneic donor bone marrow/stem cell under appropriate host

conditioning. In humans, BMT-induced mixed chimerism has been shown to confer acceptance of donor liver allografts without long-term immunosuppression. However, recipients must be able to withstand the conditioning regimens that allow donor stem cells to engraft.

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## Electroacupuncture improves gut barrier dysfunction in prolonged hemorrhagic shock rats through vagus anti-inflammatory mechanism

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### Abstract

**AIM:** To investigate whether electroacupuncture (EA) at Zusanli (ST36) prevents intestinal barrier and remote organ dysfunction following prolonged hemorrhagic shock through a vagus anti-inflammatory mechanism.

**METHODS:** Sprague-Dawley rats were subjected to about 45% of total blood volume loss followed by delayed fluid replacement (DFR) with Ringer lactate 3h after hemorrhage. In a first study, rats were randomly divided into six groups: (1) EAN: EA at non-channel acupoints followed by DFR; (2) EA: EA at ST36 after hemorrhage followed by DFR; (3) VGX/EA: vagotomy (VGX) before EA at ST36 and DFR; (4) VGX/EAN: VGX before EAN and DFR; (5)  $\alpha$ -bungarotoxin ( $\alpha$ -BGT)/EA:

intraperitoneal injection of  $\alpha$ -BGT before hemorrhage, followed by EA at ST36 and DFR; and (6)  $\alpha$ -BGT/EAN group:  $\alpha$ -BGT injection before hemorrhage followed by EAN and DFR. Survival and mean arterial pressure (MAP) were monitored over the next 12 h. In a second study, with the same grouping and treatment, cytokine levels in plasma and intestine, organ parameters, gut injury score, gut permeability to 4 kDa FITC-dextran, and expression and distribution of tight junction protein ZO-1 were evaluated.

**RESULTS:** MAP was significantly lowered after blood loss; EA at ST36 improved the blood pressure at corresponding time points 3 and 12 h after hemorrhage. EA at ST36 reduced tumor necrosis factor- $\alpha$  and interleukin (IL)-6 levels in both plasma and intestine homogenates after blood loss and DFR, while vagotomy or intraperitoneal injection of  $\alpha$ -BGT before EA at ST36 reversed its anti-inflammatory effects, and EA at ST36 did not influence IL-10 levels in plasma and intestine. EA at ST36 alleviated the injury of intestinal villus, the gut injury score being significantly lower than that of EAN group ( $1.85 \pm 0.33$  vs  $3.78 \pm 0.59$ ,  $P < 0.05$ ). EA at ST36 decreased intestinal permeability to FITC-dextran compared with EAN group ( $856.95$  ng/mL  $\pm$   $90.65$  ng/mL vs  $2305.62$  ng/mL  $\pm$   $278.32$  ng/mL,  $P < 0.05$ ). EA at ST36 significantly preserved ZO-1 protein expression and localization at 12 h after hemorrhage. However, EA at non-channel acupoints had no such effect, and abdominal vagotomy and  $\alpha$ -BGT treatment could weaken or eliminate the effects of EA at ST36. Besides, EA at ST36 decreased blood aminotransferase, MB isoenzyme of creatine kinase and creatinine vs EAN group at corresponding time points. At the end of 12-h experiment, the survival rate of the EA group was significantly higher than that of the other groups.

**CONCLUSION:** EA at ST36 attenuates the systemic inflammatory response, protects intestinal barrier integrity, improves organ function and survival rate after

hemorrhagic shock *via* activating the cholinergic anti-inflammatory mechanism.

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**Key words:** Hemorrhagic shock; Zusanli; Electro-acupuncture; Intestinal permeability; Tight junction

**Core tip:** The most important novel findings from this study are that when delayed resuscitation is inevitable during emergency situations such as hemorrhagic shock occurring in war without sufficient fluids, electroacupuncture at ST36 can be performed and it can successfully attenuate systemic inflammation, decrease gut injury and permeability and improve blood pressure and outcomes, which is consistent with preserved intestinal barrier function after hemorrhage and delayed fluid resuscitation.

Du MH, Luo HM, Hu S, Lv Y, Lin ZL, Ma L. Electroacupuncture improves gut barrier dysfunction in prolonged hemorrhagic shock rats through vagus anti-inflammatory mechanism. *World J Gastroenterol* 2013; 19(36): 5988-5999 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/5988.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.5988>

## INTRODUCTION

The current treatment for hypovolemic shock focuses on maintaining sufficient tissue perfusion and vital organ function with early and adequate fluid replenishment. Delayed fluid resuscitation for hemorrhagic shock usually occurs when mass casualties happen in austere environments such as battlefield, earthquake, or accidents, where intravenous fluid resuscitation is often difficult or even impossible. Subsequent to delayed resuscitation of hypovolemic shock, a high mortality and an increase in incidence of serious complications such as systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS) may thus befall to these victims<sup>[1]</sup>. Although delayed resuscitation have been demonstrated to result in a more profound shock insult than early resuscitation<sup>[2,3]</sup>, its pathological mechanisms remain poorly understood. One potential pathogenic mechanism appears to be associated with proinflammatory cytokine response<sup>[4,5]</sup> and, gut plays a key role in the development of intestinal and systemic inflammatory response following hemorrhagic shock and severe burn<sup>[6]</sup>. Gut becomes a source of proinflammatory mediators resulting from impairment of intestinal mucosal barrier that may amplify SIRS, produce a systemic response state and distant organ failure, and lead to MODS or even death<sup>[7-9]</sup>. Thus, interventions to prevent intestinal barrier breakdown, excessive inflammation and subsequent organ dysfunction when hemorrhagic shock prolonged due to delayed resuscitation are crucial in controlling hemorrhagic shock without sufficient fluid infusion, especially during evacuation and transportation.

The cholinergic anti-inflammatory pathway is a neural mechanism that inhibits the expression of pro-inflammatory cytokines through the interaction of the principle vagus nerve neurotransmitter, acetylcholine, and the cholinergic  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7nAChR$ ) subunit located on cytokine-expressing cells by stimulating the vagus nerve by either electrical or pharmacological methods<sup>[10,11]</sup>. Activation of the cholinergic anti-inflammatory pathway by vagus nerve stimulation can prevent cytokine release and tissue injury<sup>[12]</sup>, prolong survival and protect against the development of hypotension in rats during lethal hemorrhagic shock<sup>[13]</sup>. Recently, researchers have demonstrated an expanded role for vagus nerve stimulation and the cholinergic anti-inflammatory mechanism that provides a protective effect on the gut against epithelial barrier dysfunction and alleviates inflammatory injury in intestine and remote organs<sup>[14-16]</sup>. However, due to complicated manipulation and untoward side effects, including serious tissue injury, it is still difficult to apply electrical stimulation to the vagus nerve in clinical practice. Therefore, a more clinically desirable alternative therapy needs to be established during the resuscitative phase of trauma care.

Acupuncture as one of the therapeutic maneuvers in traditional Chinese medicine (TCM) has been applied in clinics for thousands of years, and it has been found to have a bidirectional neuron-endocrine-immune system regulating effect, and antagonize systemic inflammatory response without side effects. We have demonstrated that electroacupuncture (EA) at ST36 had a significantly positive effect on hemorrhagic shock<sup>[2]</sup> in rats with delayed fluid resuscitation, however, its mechanism remains unknown. We have recently furthermore proved that EA alleviated intestinal barrier insult and system inflammation in a rat ischemia model through activating the cholinergic anti-inflammatory pathway<sup>[17]</sup>. Therefore, we investigated whether EA at ST36 protected intestinal barrier function, thus preventing remote organ injury after prolonged hemorrhagic shock in rats with delayed fluid replacement (DFR) through activating the cholinergic anti-inflammatory-dependent mechanism.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats (8-10 wk, 240-260 g) were purchased from Experimental Animal Center of Military Medical Sciences of the Chinese PLA. Rats were acclimatized for a while in mesh cages in a temperature-controlled room with a 12-h light-dark cycle in the animal quarter of our laboratory and fasted overnight, but allowed free access to water until 4 h before surgery. The research protocols were approved by the Committee of Scientific Research of the First Hospital Affiliated to General Hospital of PLA, China. The experiment was conducted in compliance with the Guide for Care and Use of Laboratory Animals of National Research Council, China.

### Surgical procedures

Rats were anesthetized and instrumented with 3% isoflurane inhalation (Yeeran Technology Limited, Beijing, China). Ketamine 10 mg/kg was hypodermically injected for local anesthesia. Isoflurane (0.7%) was used to maintain anesthesia during the experiments. Animals were allowed to breath spontaneously under a nose cone scavenging system using a veterinary anesthesia delivery system (Kent Scientific TOPO, Torrington, CT, United States). With aseptic technique, poly-ethylene (PE50) catheters were placed in the right carotid artery for continuous artery blood pressure monitoring, in the left femoral artery for blood withdrawal, and in the right femoral vein for fluid infusion. A 2-cm upper-midline laparotomy incision was made to identify gastroesophageal junction and expose the dorsal and ventral vagus nerve on the distal esophagus with a Phenix XLT165-LB stereomicroscope (Phenix Optical Instrument Group Company, Jiangxi Province, China). Rectal temperature was maintained at 37 °C with a heating pad and a heating lamp.

### Hemorrhagic shock protocol

Each animal's estimated blood volume was calculated using the formula<sup>[18]</sup>: Total blood volume (TBV) (mL) = body weight (g) × 0.06 (mL/g) × 0.77. Hemorrhagic shock was induced by withdrawing 45% of the calculated TBV within 20 min (30% was withdrawn over the first 3 min and suspended for 7 min, and the rest 15% was withdrawn over another 10 min), using an infusion or a withdrawal pump (Kelifeng Apparatus, Beijing, China). The completion of the hemorrhagic shock model concluded the preparation phase, and the time was metered 0. Mean arterial pressure (MAP) was monitored using a PICCO-PLUS cardio-pulmonary volume monitor (PULSION, Feldkirchen, Germany) after exsanguinations were initiated, and recorded 0.5 h before bleeding, immediately upon completion of the hemorrhagic shock (0), 3 h and 12 h after blood loss. The early survival rate was recorded at 12 h after bleeding. Fluid resuscitation was initiated 3 h after exsanguinations.

### Animal grouping and treatment

All the animals underwent the same surgical procedure and hemorrhagic shock protocol, and the experimental rats were randomly assigned to six groups: (1) EAN group: Rats underwent EA at non-channel acupoints located 0.5 cm lateral and distal from ST36 points<sup>[19]</sup>, followed by DFR similar to EA group; EA parameters and delayed rehydration were the same as the EA group; (2) EA group: Animals underwent EA at ST36 points, which were located at posterior and lateral side of the knee joint, 5 mm below capitulum fibulae<sup>[19]</sup>, immediately after the blood loss followed by DFR 3 h after hemorrhage. EA at ST36 with an electro-acupuncture apparatus (HANS, LH202H) was performed as described before<sup>[17]</sup>. Briefly, both hind limbs were shaved and the skin was disinfected. ST36 acupuncture point was punctured with a depth of 7 mm, and then the needle was connected

with an electro-acupuncture apparatus. The electric current with the intensity of 2 mA and 2-100Hz was continued for 1.5 h immediately after hemorrhage. Three hours after blood loss, rats were given a femoral vein infusion with Ringer lactate (2 times the amount of blood loss); (3) VGX/EA group: Animals underwent vagotomy of the dorsal and ventral vagus nerve on the distal esophagus prior to EA at ST36 immediately after blood loss followed by DFR; EA parameters and delayed rehydration were the same as the EA group; (4) VGX/EAN group: Animals underwent vagotomy similar to VGX/EA group before EA at non-channel acupoints similar to EAN group immediately after blood loss followed by DFR. EA parameters and delayed rehydration were the same as the EA group; (5)  $\alpha$ -BGT/EA group:  $\alpha$ -bungarotoxin ( $\alpha$ -BGT 1  $\mu$ g/kg, an antagonist of  $\alpha$ 7 subunit of cholinergic nicotinic receptor, which inhibits the  $\alpha$ 7 subunit of acetylcholine receptors by blocking a pivotal communication pathway between the efferent vagus and intestinal immune cells<sup>[20,21]</sup>) was injected intraperitoneally prior to hemorrhage and followed by EA at ST36 and DFR similar to EA group. EA parameters and delayed rehydration were the same as the EA group; and (6)  $\alpha$ -BGT/EAN group:  $\alpha$ -BGT was injected intraperitoneally prior to hemorrhage and followed by EA at non-channel acupoints and DFR similar to EAN group.

Each group was then randomly divided into two subgroups: one subgroup ( $n = 12$ ) for investigation of survival rate and MAP; the other one ( $n = 18$ ) for cytokine levels, organ parameters, gut injury score, ZO1 detection and intestinal permeability to FITC-dextran test; blood and intestine for cytokine levels were harvested at 0, 3 and 12 h after blood loss (3-5 animals per group); blood for organ parameters test, and intestine for gut injury score, ZO1 detection and intestinal permeability to FITC-dextran test were harvested at 12 h after blood loss (3-5 animals per group).

The scheme for whole experiment is as follows in Figure 1.

### Samples of blood and intestine

Rats were anesthetized with 3% isoflurane inhalation. Blood was drawn through left femoral artery at 0, 3 and 12 h after blood loss, and then the animals were sacrificed for distal small intestine harvest. Plasma was obtained by centrifuging the blood at  $10000 \times g$  for 10 min at 4 °C. Organ functions were assessed by measuring blood aminotransferase (ALT), MB isoenzyme of creatine kinase (CK-MB) and creatinine (Cr) using a Cobas 6000 automatic biochemical analyzer (Roche Diagnostics, Basel, Switzerland). Segments of distal small intestine were harvested and immediately homogenized on ice with 1 mL denaturing lysis buffer or nondenaturing lysis buffer for Western blotting or ELISA. The homogenate was then centrifuged at  $10000 \times g$  for 10 min at 4 °C. Aliquots of the supernatants of plasma and tissue were stored at -80 °C until use. Segments of intestine were also harvested and fixed in 4% paraformaldehyde for histologic

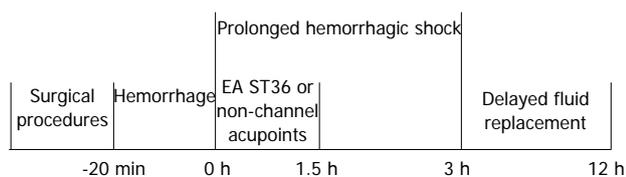


Figure 1 The scheme for whole experiment. EA: Electroacupuncture; ST36: Zusanli.

evaluation and immunofluorescence.

### Detection of TNF- $\alpha$ , IL-6 and IL-10 levels in plasma and intestine

TNF- $\alpha$ , IL-6 and IL-10 levels in the plasma and intestine were assessed using commercially available ELISA kit according to the protocol provided by the manufacturer (Nanjing Jiancheng Corp., Nanjing, China). Supernatants were transferred into fresh tubes for the evaluation. Briefly, after adding 50  $\mu$ L assay buffer, 50  $\mu$ L samples or standard concentration for TNF- $\alpha$ , IL-6 or IL-10 were incubated with 50  $\mu$ L diluted Biotin-Conjugate for 2 h at room temperature. After 3 washes, the plates were incubated with Streptavidin-HRP for 1 h at room temperature. After 3 washes, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to the plates for 15 min and the reaction was stopped with stop solution. The absorbance rate was read at 450 nm. The concentrations of the samples were calculated according to the standard curve. The plasma TNF- $\alpha$ , IL-6 and IL-10 levels were expressed as pg/mL. Intestine TNF- $\alpha$ , IL-6 and IL-10 levels were expressed as picograms per milligram of protein.

### Histopathologic score

The paraformaldehyde-fixed intestine was embedded in paraffin, and cut into 2- $\mu$ m sections. Hematoxylin and eosin staining of the intestine was performed by the Pathology Department of the First Hospital Affiliated to the People's Liberation Army General Hospital. Then the sections were viewed under a light microscope and evaluated by a pathologist blinded to the experimental groups. The injury to the intestinal mucosa was scored using the grading system as previously described<sup>[22]</sup>.

### Intestinal epithelial permeability

An *in vivo* intestinal permeability assay was performed to assess gut barrier function as described by Kao *et al.*<sup>[23]</sup>. Briefly, 30 min before sacrifice, animals were anesthetized with inhaled isoflurane. A midline laparotomy incision was made and a 10-cm segment of distal ileum was isolated between silk ties. A solution of 1.0 mL phosphate-buffered saline (PBS, pH 7.2) containing 25 mg 4-kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, St. Louis, MO, United States) was injected into the lumen of the isolated segment of intestine. The bowel was returned to the abdominal cavity and the abdomen was closed. Animals were maintained lightly under general anesthesia for 30 min, and systemic blood was drawn by left femoral artery puncture and placed in heparinized

Eppendorf tubes on ice. Plasma was obtained by centrifuging the blood at  $10000 \times g$  for 10 min at  $-4^\circ\text{C}$ . Plasma fluorescence was measured by a fluorescence spectrophotometer (Synergy2; BioTek Multi-Detection Microplate reader, United States) and compared with a standard curve of known concentrations of FITC-dextran diluted in rat plasma.

### Immunofluorescence

After deparaffinization, the intestine sections were rehydrated and incubated in citrate buffer (Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for heat-induced antigen retrieval. After three washes with PBS, sections were incubated with 3% bovine serum albumin (BSA) (Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 30 min to block nonspecific binding sites. The sections were then incubated in the ZO-1 antibody (1:100; Life Technologies, Gaithersburg, MD, United States) at  $4^\circ\text{C}$  overnight. The following day, after washing with PBS three times, they were treated with Alexa Fluor 488 secondary goat anti-rabbit antibody in 1% BSA for 1 h at room temperature. Prolong Fade (Antifade Mounting Medium, Beyotime Institute of Biotechnology, Beijing, China) was added on placement of cover slips. Images were viewed using the Olympus fluorescence microscope (BX51-DP71) with exposure-matched settings.

### ZO-1 expression

The harvested gut tissues were placed in 1 mL lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% NP-40; 0.1% sodium dodecyl sulphate), then homogenized and centrifuged at  $12000 \times g$  for 10 min. Following centrifugation, the supernatant was collected and analyzed for protein concentration. Protein concentrations were determined using a protein assay kit (Applygen Technologies Inc, Beijing, China). Total protein (100  $\mu$ g) was loaded onto a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and run at 120 volts for 2 h. After electrophoresis, the protein was transferred to a polyvinylidene difluoride membrane (PVDF; Applygen Technologies Inc, Beijing, China) and blocked for 2 h in TBST (50 mmol/L Tris; 150 mmol/L NaCl; 0.05% Tween 20) containing 5% milk (Applygen Technologies Inc, Beijing, China). The membrane was then incubated with the primary antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000; Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China), and ZO-1 (1:500; Life Technologies, Gaithersburg, MD, United States) at  $4^\circ\text{C}$  overnight. After 3 washes in TBST, the membrane was incubated with corresponding secondary antibodies conjugated to horseradish peroxidase at room temperature for 30 min and chemiluminescence was detected using SuperECL Plus (Applygen Technologies Inc, Beijing, China). Films were developed using a standard photographic procedure. Quantitative analysis of detected bands was carried out by densitometer scanning (ImageJ).

### Statistical analysis

Data were analyzed using a commercial statistical soft-

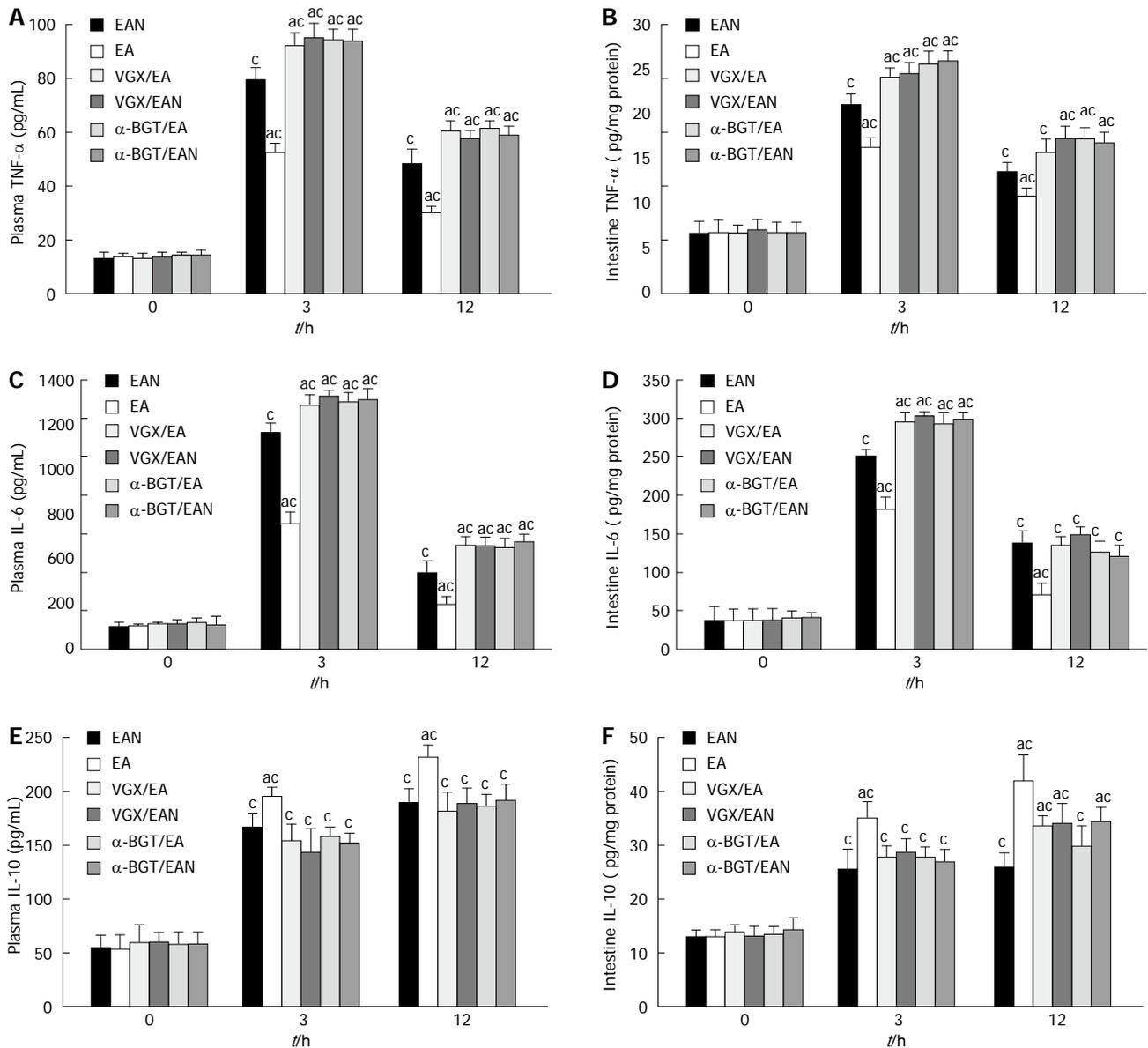


Figure 2 Tumor necrosis factor- $\alpha$ , interleukin-6 and interleukin-10 levels in plasma and intestine at 0, 3 and 12 h after blood loss. Blood samples and intestine were obtained at 0, 3 and 12 h after blood loss. Data are expressed as mean  $\pm$  SD (3-5 animals per group at each time point). <sup>a</sup> $P < 0.05$  vs EAN group; <sup>c</sup> $P < 0.05$  vs 0 h in the same group. EA: Electroacupuncture; VGX: Vagotomy;  $\alpha$ -BGT:  $\alpha$ -bungarotoxin; TNF: Tumor necrosis factor; IL: Interleukin.

ware package (SPSS statistics 17.0). Continuous variables were expressed as mean  $\pm$  SEM. Statistical significance of differences between groups was determined using one way analysis of variance (ANOVA) followed by Dunnett's test and SNK-q for multiple comparisons. If some variables were abnormally distributed, the Kruskal-Wallis H test was used. Significance was declared for  $P$  values  $< 0.05$ .

## RESULTS

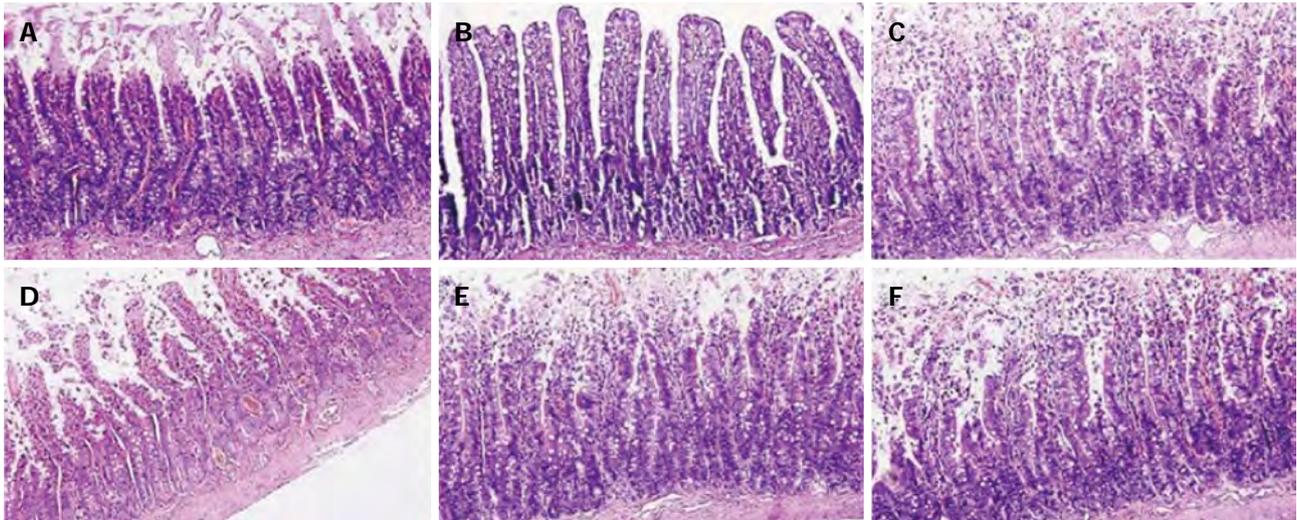
### Effect of EA ST36 on plasma and intestinal cytokine levels

Figure 2 illustrates the effect of EA ST36 on TNF- $\alpha$  (Figure 2A, B), IL-6 (Figure 2C, D) and IL-10 (Figure 2E, F) levels in plasma and intestine in rats with DFR after

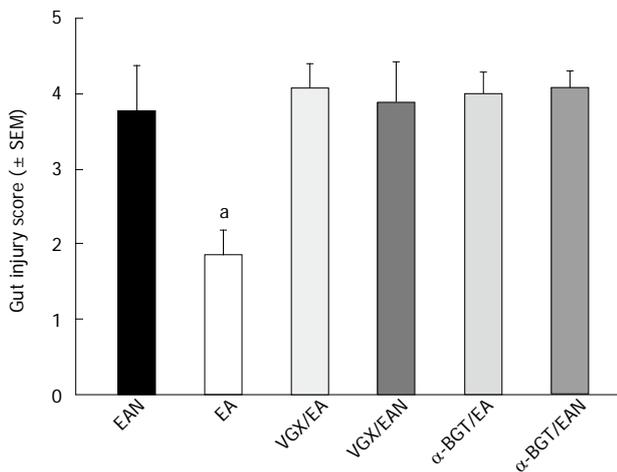
fatal hemorrhagic shock. Hemorrhage and DFR induced pronounced rises in the concentrations of TNF- $\alpha$ , IL-6 and IL-10 in plasma and intestine homogenates. EA at ST36 reduced TNF- $\alpha$  and IL-6 levels in both plasma and intestine homogenates after blood loss and DFR, while vagotomy or intraperitoneal injection of  $\alpha$ -BGT before EA at ST36 reversed its anti-inflammatory effects. In contrast, EA at ST36 did not influence the increases in plasma and intestinal IL-10 during hemorrhagic shock and DFR. These evidences suggested that EA at ST36 attenuated the release of TNF- $\alpha$  and IL-6, but did not suppress IL-10 level in plasma and intestine.

### EA ST36 decreased intestinal injury

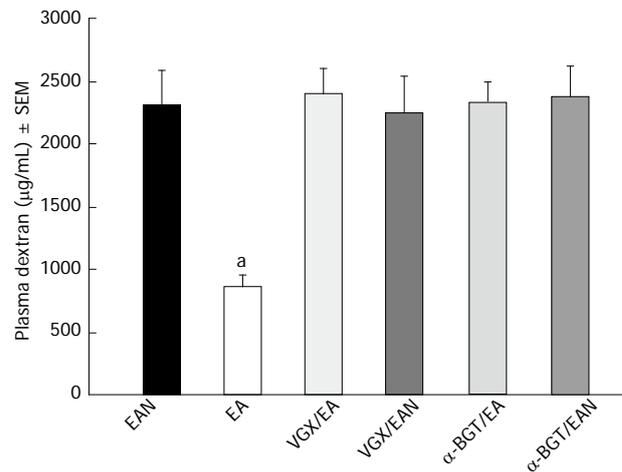
Histological evaluation of intestinal injury was performed at 12 h after blood loss. Sections of distal ileum from ani-



**Figure 3** Intestinal histology at 12 h after blood loss. Electroacupuncture (EA) at ST36 protected against intestinal injury after hemorrhagic shock and delayed fluid replacement, whereas EA at ST36 after vagotomy or injection of  $\alpha$ -bungarotoxin eliminated such protection. Sections of distal ileum were harvested at 12 h after blood loss and stained with hematoxylin and eosin. All images are taken at  $\times 200$  magnification with black bar = 5  $\mu$ m (3-5 animals per group at 12 h after blood loss).



**Figure 4** Gut injury scores at 12 h after blood loss. Gut injury was scored by a pathologist blinded to the experimental groups on a scale of 0-4, (as described in Materials and Methods). <sup>a</sup> $P < 0.05$  vs EAN group, (3-5 animals per group at 12 h after blood loss). EA: Electroacupuncture; VGX: Vagotomy;  $\alpha$ -BGT:  $\alpha$ -bungarotoxin.



**Figure 5** Intestinal permeability to 4-kDa fluorescein isothiocyanate-dextran at 12 h after blood loss. Electroacupuncture (EA) at ST36 protected the intestine from an increase in permeability after hemorrhagic shock and delayed fluid replacement, whereas EA at ST36 after vagotomy or injection of  $\alpha$ -bungarotoxin eliminated such protection. <sup>a</sup> $P < 0.05$  vs EAN group, (3-5 animals per group at 12 h after blood loss). EA: Electroacupuncture; VGX: Vagotomy;  $\alpha$ -BGT:  $\alpha$ -bungarotoxin

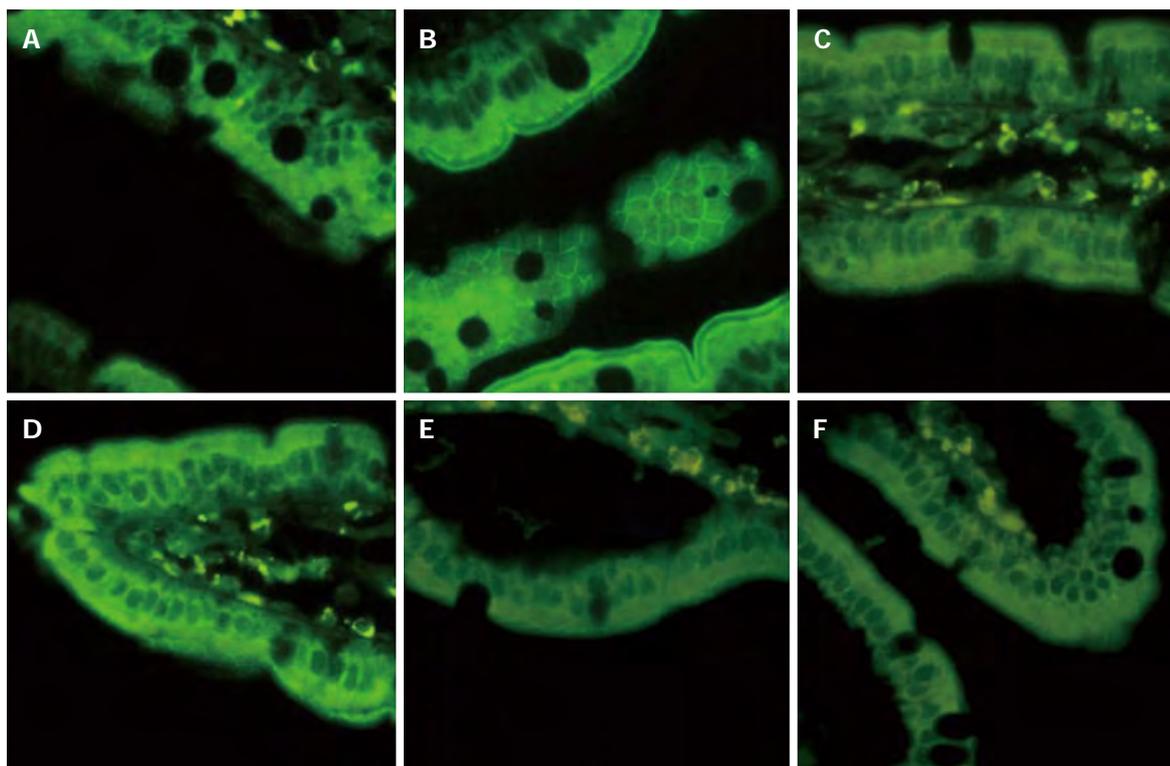
mals in EAN group showed villous tip necrosis, blunting, and sloughing of villi (Figure 3A). EA at ST36, which was applied immediately after blood loss, significantly attenuated the mucosal damage (Figure 3B). In contrast, when abdominal vagotomy or intraperitoneal injection of  $\alpha$ -BGT was performed and as such the intact neuroenteric axis was interrupted, EA at both ST36 and non-channel acupoints failed to prevent the histologic changes induced by hemorrhagic shock in the gut (Figure 3C-F). These data demonstrated that an intact vagus nerve is required for the biological effect of EA at ST36 in protecting against gut injury.

Gut injury scores were measured in all 6 groups (Figure 4). Animals in EAN group had an average injury score that was significantly higher than in EA group (3.78  $\pm$  0.59 *vs* 1.85  $\pm$  0.33,  $P < 0.05$ ). Abdominal vagotomy

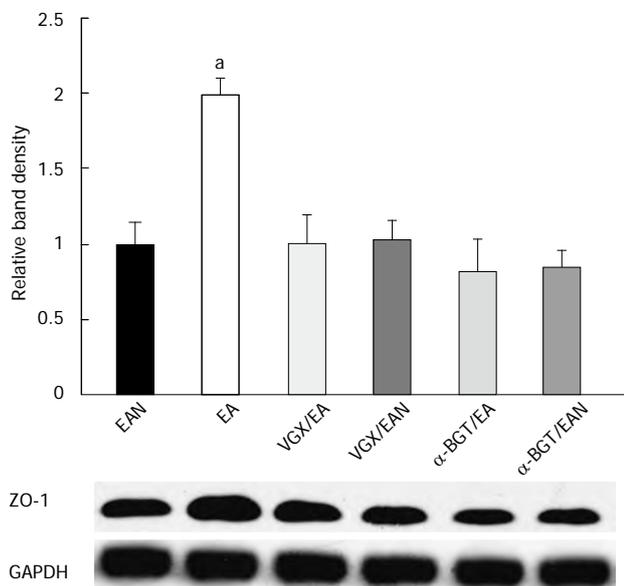
or intraperitoneal injection of  $\alpha$ -BGT before EA at ST36 eliminated the protective effects of EA at ST36 and resulted in similar scores as animals in VGX/EAN,  $\alpha$ -BGT/EAN and EAN groups (3.9  $\pm$  0.53, 4.1  $\pm$  0.29 *vs* 3.78  $\pm$  0.59;  $P > 0.05$ , respectively).

#### EA at ST36 lowered intestinal permeability

The intestinal permeability was evaluated in an *in vivo* assay using FITC-Dextran (Figure 5). Animals in EA group had a significantly lower level of plasma FITC-Dextran when compared with EAN group (856.95  $\pm$  90.65 ng/mL *vs* 2305.62  $\pm$  278.32 ng/mL,  $P < 0.05$ ). However, when abdominal vagotomy or  $\alpha$ -BGT injection was performed before EA at ST36, the intestinal permeability



**Figure 6** Intestinal ZO-1 immunofluorescent staining at 12 h after blood loss. Animals in EAN group showed a low fluorescent intensity at the cell periphery after hemorrhagic shock, and electroacupuncture (EA) at ST36 showed preservation of the robust structure of ZO-1 staining, whereas after vagotomy or injection of  $\alpha$ -bungarotoxin, it eliminated such protection. All images are taken at  $\times 400$  magnification with black bar = 5  $\mu\text{m}$ . (3-5 animals per group at 12 h after blood loss, size bar = 2  $\mu\text{m}$ ).



**Figure 7** Intestinal ZO-1 protein expression at 12 h after blood loss. Intestinal extracts were obtained from animals at 12 h after blood loss for measurement of ZO-1 protein expression using Western blotting. Representative Western blotting for the ZO-1 protein is shown with its corresponding glyceraldehyde 3-phosphate dehydrogenase loading control to demonstrate equal protein load in all lanes. Electroacupuncture at ST36 resulted in preservation of protein expression. Significant reduction in ZO-1 expression was seen in all the other groups.  $^aP < 0.05$  vs EAN group, (3-5 animals per group at 12h after blood loss). EA: Electroacupuncture; VGX: Vagotomy;  $\alpha$ -BGT:  $\alpha$ -bungarotoxin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

was indistinguishable from animals in EAN group, and animals in VGX/EAN or  $\alpha$ -BGT/EAN group also showed no protection in reducing intestinal permeability compared with animals in EAN group ( $2249.87 \pm 294.17$  ng/mL and  $2400.15 \pm 203.15$  ng/mL vs  $2305.62 \pm 278.32$  ng/mL).

**EA ST36 prevented loss and redistribution of ZO-1**

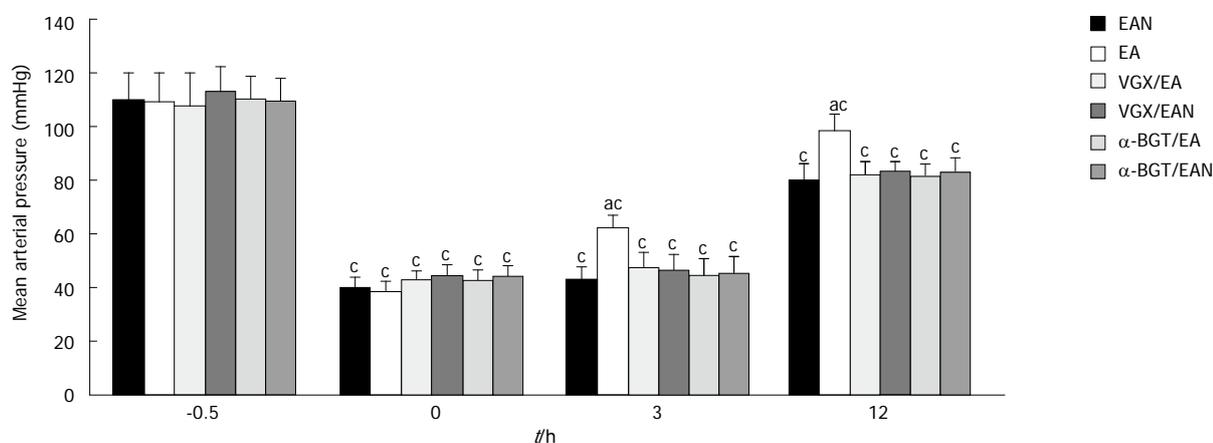
The tight junction protein, ZO-1, undergoes protein expression alterations in response to hemorrhagic shock. Exposure-matched fluorescent intensity correlated to the amount of ZO-1 protein expression after immunostaining (Figure 6). After blood loss, animals in EAN group showed a loss and redistribution in ZO-1 expression evidenced by a low fluorescent intensity at the cell periphery (Figure 6A). Animals in EA group (Figure 6B) showed preservation of the robust structure of ZO-1. In contrast, animals treated with EA at ST36 or non-channel acupoints after abdominal vagotomy, no protection was afforded to the intestinal mucosa, evidenced by the easy interruption and partial disappearance of ZO-1 staining in the periphery of villous epithelial cells (Figure 6C, D). And EA at ST36 or non-channel acupoints after  $\alpha$ -BGT injection also offered no protection against ZO-1 disruption (Figure 6E, F).

These results were confirmed by Western blotting for the ZO-1 protein in intestinal tissue lysates (Figure 7). When compared with the average relative band density of

**Table 1** Alanine aminotransferase, MB isoenzyme of creatine kinase and creatinine of rats of all groups after hemorrhagic shock with delayed fluid replacement

Variables	EAN	EA	VGX/EA	VGX/EAN	$\alpha$ -BGT/EA	$\alpha$ -BGT/EAN
ALT ( $\mu$ /L)						
0 h	34.0 $\pm$ 3.4	33.2 $\pm$ 1.3	35.9 $\pm$ 2.9	36.1 $\pm$ 1.2	32.7 $\pm$ 5.4	32.8 $\pm$ 4.1
3 h	76.7 $\pm$ 10.1 <sup>c</sup>	46.6 $\pm$ 5.7 <sup>ac</sup>	78.6 $\pm$ 9.7 <sup>c</sup>	79.5 $\pm$ 11.2 <sup>c</sup>	81.2 $\pm$ 8.5 <sup>c</sup>	81.4 $\pm$ 9.2 <sup>c</sup>
12 h	63.0 $\pm$ 7.9 <sup>c</sup>	38.1 $\pm$ 7.1 <sup>ac</sup>	65.1 $\pm$ 10.3 <sup>c</sup>	67.5 $\pm$ 9.7 <sup>c</sup>	62.4 $\pm$ 11.6 <sup>c</sup>	63.7 $\pm$ 12.3 <sup>c</sup>
CK-MB ( $\mu$ /L)						
0 h	112 $\pm$ 21	110 $\pm$ 17	113 $\pm$ 18	114 $\pm$ 21	112 $\pm$ 15	119 $\pm$ 22
3 h	468 $\pm$ 32 <sup>c</sup>	232 $\pm$ 25 <sup>ac</sup>	459 $\pm$ 37 <sup>c</sup>	469 $\pm$ 33 <sup>c</sup>	477 $\pm$ 32 <sup>c</sup>	469 $\pm$ 27 <sup>c</sup>
12 h	365 $\pm$ 49 <sup>c</sup>	184 $\pm$ 32 <sup>ac</sup>	333 $\pm$ 42 <sup>c</sup>	351 $\pm$ 47 <sup>c</sup>	378 $\pm$ 48 <sup>c</sup>	398 $\pm$ 51 <sup>c</sup>
Cr ( $\mu$ mol/L)						
0 h	23.8 $\pm$ 3.3	23.1 $\pm$ 4.5	23.4 $\pm$ 4.4	24.1 $\pm$ 2.7	23.4 $\pm$ 3.6	23.2 $\pm$ 3.7
3 h	59.2 $\pm$ 9.2 <sup>c</sup>	41.5 $\pm$ 3.3 <sup>ac</sup>	58.9 $\pm$ 8.4 <sup>c</sup>	62.1 $\pm$ 9.5 <sup>c</sup>	64.8 $\pm$ 10.7 <sup>c</sup>	65.7 $\pm$ 11.2 <sup>c</sup>
12 h	48.1 $\pm$ 5.3 <sup>c</sup>	30.2 $\pm$ 4.2 <sup>ac</sup>	48.7 $\pm$ 4.6 <sup>c</sup>	48.9 $\pm$ 3.6 <sup>c</sup>	48.2 $\pm$ 5.2 <sup>c</sup>	48.5 $\pm$ 4.5 <sup>c</sup>

<sup>a</sup> $P < 0.05$  vs EAN group; <sup>c</sup> $P < 0.05$  vs 0 h in the same group, (3-5 animals per group at each time point after blood loss). ALT: Alanine aminotransferase; CK-MB: MB isoenzyme of creatine kinase; Cr: Creatinine; EA: Electroacupuncture; VGX: Vagotomy;  $\alpha$ -BGT:  $\alpha$ -bungarotoxin.



**Figure 8** Effect of electroacupuncture ST36 on mean arterial pressure in rats after hemorrhagic shock with delayed fluid replacement. <sup>a</sup> $P < 0.05$  vs EAN group; <sup>c</sup> $P < 0.05$  vs 0 h in the same group, (3-5 animals per group at 12 h after blood loss). EA: Electroacupuncture; VGX: Vagotomy;  $\alpha$ -BGT:  $\alpha$ -bungarotoxin.

animals in EAN group, animals treated with EA at ST36 had significantly higher ZO-1 expression ( $P < 0.05$ ). In contrast, intestinal ZO-1 protein levels were significantly decreased in animals receiving abdominal vagotomy or  $\alpha$ -BGT injection before EA at ST36. ZO-1 protein levels in VGX/EAN or  $\alpha$ -BGT/EAN group receiving EA at non-channel acupoints after vagotomy or  $\alpha$ -BGT injection were not significantly different from that in EAN group.

#### EA ST36 lowered plasma ALT, CK-MB and Cr

There was a significant decrease in ALT, CK-MB and Cr in the EA group compared with EAN group ( $P < 0.05$ ; Table 1). However, there was no significant difference of ALT, CK-MB, BUN and Cr among the EAN, VGX/EA, VGX/EAN,  $\alpha$ -BGT/EA and  $\alpha$ -BGT/EAN groups.

#### EA at ST36 improved the blood pressure and raised the survival rate

MAP in each group was significantly lowered after blood loss. MAP at time 0 was only 35%-45% of that before blood loss; after 3 h, MAP in each group increased to dif-

ferent degrees. At 3 h and 12 h after hemorrhage, the EA group displayed higher MAP levels than the EAN group ( $P < 0.05$ ) (Figure 8) while the VGX/EA, VGX/EAN,  $\alpha$ -BGT/EA and  $\alpha$ -BGT/EAN groups had low MAP levels similar to the EAN group.

As shown in our previous study<sup>[2]</sup>, at 12 h after blood loss, the survival rate of the EA group was significantly higher than that of the other groups ( $P < 0.05$ ). Ten (83.3%) of 12 rats in the EA group were alive 12 h after blood loss. In contrast, only 5 (41.7%) of 12 rats in the EAN group survived after being subjected to hemorrhagic shock treated with EA at non-channel acupoints and DFR. When rats subjected to hemorrhagic shock were treated with EA at ST36 after abdominal vagotomy or  $\alpha$ -BGT injection, 6 (50%) of 12 rats in VGX/EA group, 5 (41.7%) in  $\alpha$ -BGT/EA group, 5 (41.7%) in VGX/EAN group and 5 (41.7%) in  $\alpha$ -BGT/EAN group survived 12 h after blood loss.

## DISCUSSION

The most important novel findings from this study

are that when delayed resuscitation is inevitable during emergency situations such as hemorrhagic shock in war without sufficient fluids, EA at ST36 can be performed and it can successfully attenuate systemic inflammation, decrease gut injury and permeability and improve blood pressure and outcomes, which is consistent with preserved intestinal barrier function after hemorrhage and delayed fluid resuscitation. Based on our previous studies from this lab<sup>[2,24]</sup>, these results have provided further evidence for a role of acupuncture in an emergency treatment after shock and trauma.

It is regarded that the intestinal tract is one of the earlier organs involved in ischemia-reperfusion injury after hemorrhagic shock. In order to maintain the blood supply of the vital organs during hemorrhagic shock, the intestinal blood flow sharply reduced, and dysfunction of the intestinal mucosal barrier occurs. The ischemia of the small intestine leads rapidly to an impairment of mucosa barrier function, and thus the earliest restitution of the mesenteric blood flow is essential. However, in a very harsh environment with a lack of decent medical support, such as accident and war, immediate resuscitation is sometimes unavailable, the delayed fluid resuscitation occurs, threatening the life of those with extensive injury or hemorrhagic shock. It has also been recognized that a delay in such replenishment could sometimes be fatal due to complications subsequent to delayed resuscitation of hypovolemic shock. One strategy for reducing such a hazard is to try to supplement liquid with sufficient electrolytes by mouth until intravenous infusion fluids are available<sup>[25]</sup>. In recent years, we have been working on the alternative methods to treat the complications caused by delayed fluid resuscitation. We have found that oral resuscitation is an effective way to partly replace immediate resuscitation when the intravenous infusion is not available<sup>[1]</sup>. More recently, we have demonstrated that EA at ST36 can significantly improve the survival rate and blood pressure after fatal hemorrhagic shock in rats<sup>[2]</sup>.

The gut barrier has been found to be seriously damaged at the early phase of hemorrhagic shock<sup>[26]</sup>. The protective effect of traditional Chinese medicine against intestinal and gastric mucosal injury after hemorrhagic shock in rats has been investigated<sup>[27,28]</sup>. Increasing evidences suggest that the effect of EA at ST36 for gastrointestinal disorders may involve vagal reflex. The dorsal vagal complex (DVC) consists of the nucleus of the solitary tract (NTS), which receives primary visceral afferent information, and the dorsal motor nucleus of the vagus (DMV), which contains the efferent vagal neurons innervating visceral organs. Therefore, DVC plays an important role in regulating visceral functions. A previous study has demonstrated that there is a commonality of central nervous system (CNS) cell groups in brain controlling ST36 point, including DMV and NTS<sup>[29]</sup>. EA at ST36 generated an increased c-Fos expression in the neurons of DMV<sup>[30]</sup> and promoted the gastric myoelectric activity, which was regulated by the vagus, and substance P (SP) which is widely present in DVC and involved in the excit-

atory effects<sup>[31]</sup>. It has been proved that EA at ST36 can increase the efferent activity of the vagal nerve<sup>[32]</sup>. Taken together, these data suggest that EA at ST36 is relevant to vagus nerve and it has beneficial effects in intestinal barrier function. In this set of experiments, we stimulated ST36 points after hemorrhagic shock, established its efficacy, and compared its effects with EA at ST36 or non-channel acupoints after abdominal vagotomy or  $\alpha$ -BGT injection. We demonstrated that EA at ST36 is effective in preventing intestinal barrier breakdown after hemorrhagic shock with delayed resuscitation. We also showed, as a proof of concept, that its biological effect is dependent on an intact vagus nerve.

Increasing evidence points to extensive cross-talk between intestinal barrier breakdown and cytokine overproduction. The gut has been shown to be a source of inflammatory cytokine with capability of priming neutrophils after ischemia injury or hemorrhagic shock<sup>[6,33]</sup>. These cytokines originating from the gut may then exacerbate the systemic inflammatory response and potentially lead to a further damage to gut permeability<sup>[7,34]</sup>. It has also been demonstrated that IL-6 is essential for the development of gut barrier dysfunction after hemorrhagic shock<sup>[35]</sup>. IL-10 has also been proved to play a pivotal role in regulating proinflammatory cytokine release following trauma-hemorrhage<sup>[36]</sup>. In our previous studies, we demonstrated that EA at ST36 can alleviate intestinal pro-inflammatory factors, tissue edema and insult of intestinal mucosa<sup>[37]</sup>, significantly protect against tumor necrosis factor- $\alpha$  induced-multiple organ dysfunction in rats with sepsis<sup>[38]</sup>, and promote gastric emptying in rats with a 40% blood volume loss<sup>[39]</sup>. EA at ST36 has been shown to have a regulatory effect on TNF- $\alpha$  level in rats<sup>[40]</sup>. This study also demonstrated that acupuncture at ST36 significantly attenuated the expression of pro-inflammatory cytokine TNF- $\alpha$  and IL-6 levels in plasma and intestinal homogenate, but failed to suppress the anti-inflammatory cytokine IL-10 levels in plasma and intestine. These data suggests the anti-inflammatory potential of the use of ST36 acupuncture against hemorrhagic shock. Non-channel acupoints acupuncturing, vagotomy or  $\alpha$ -BGT injection before EA at ST36 showed no effects in reducing the production of TNF- $\alpha$  and IL-6, which suggested that anti-inflammatory effect of EA at ST36 may be exerted through the vagal nerves, and their integrity is essential.

In our previous study, we demonstrated that EA at ST36 can effectively protect organ function<sup>[24]</sup>, improve the early survival rate, increase the intestinal tissue diamine oxidase activity and alleviate intestinal ischemia in rats with DFR after hemorrhagic shock<sup>[2]</sup>, but its mechanism is unknown. This study demonstrated that this protective effect of EA at ST36 may be related to its protection of intestinal barrier function. In this study, EA at ST36 effectively prevented histologic injury of the gut mucosa, reduced permeability of the distal ileum to 4-kDa FITC-dextran and maintained intestinal tight junction protein expression and function. We also proved that this biologi-

cal effect is dependent on an intact vagus nerve. Disrupting the neurenteric axis *via* surgical abdominal vagotomy would abolish the protective effect of EA at ST36.

It has been found that tight junction proteins are critical structural proteins to maintain the mucosal barrier function<sup>[41,42]</sup>, and loss of gut barrier function is a major contributor to the systemic inflammatory response that ultimately leads to multiple organ failure<sup>[43]</sup>. Modulation of tight junction proteins can be effective in protecting the remote organ function in burned mice<sup>[44]</sup>, but its mechanism is also unclear. In this study, EA at ST36 can also significantly improve blood pressure, parameters of organ function and survival rate, which is in accordance with decreased intestinal permeability and improved intestinal tight junction protein distribution and expression.

There are several limitations in this study. All of our measures were evaluated with the rats under anesthesia, which might be different between human and animals. We did not monitor the change of vagal nerve activity before and after EA at ST36. The experimental duration is short. Meanwhile, more *in vitro* researches are needed to clarify the mechanism of ZO1 protein affected by EA at ST36. Besides, the model we used in this study is subjected to withdraw about 45% of the calculated TBV and such a large volume may be lethal and cause severe damage to the animals.

In summary, this study showed that EA at ST36 attenuated the release of TNF- $\alpha$  and IL-6 in plasma and intestine, alleviated the impairment of intestinal barrier function, and improved parameters of organ function and the early survival rate of hemorrhagic shock rats with DFR. The protective role of EA at ST36 is possibly related to an intact vagus nerve and it might exert its effects *via* cholinergic  $\alpha 7$  nicotinic acetylcholine receptor.

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## COMMENTS

### Background

Fluid resuscitation for hemorrhagic/burn shock is often challenging when mass casualties occurring in austere circumstances such as in the battlefield or site of an unexpected accident or a disaster, where intravenous fluid resuscitation is difficult or even impossible. Subsequent to delayed resuscitation of hypovolemic shock, a high mortality and an increase in incidence of serious complications such as systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS) may thus befall to these victims. Thus, interventions such as drug or acupuncture to prevent excessive inflammation and subsequent organ dysfunction when hemorrhagic shock prolonged due to delayed resuscitation are of great significance in controlling hemorrhagic shock without sufficient fluid infusion, especially during evacuation and transportation.

### Research frontiers

The current treatment for hypovolemic shock focuses on maintaining sufficient tissue perfusion and vital organ function with early and adequate fluid

replenishment. It is of great importance to seek clinically alternative therapies when delayed fluid replenishment is inevitable. Activation of the cholinergic anti-inflammatory pathway by vagus nerve stimulation has been demonstrated to inhibit inflammatory response, prolong survival and protect against the development of hypotension during lethal hemorrhagic shock. Gut, especially the intestinal barrier function, plays a key role in the development of intestinal and systemic inflammatory response and protection of organ function following hemorrhagic shock. Recently, researchers have demonstrated an expanded role for vagus nerve stimulation and the cholinergic anti-inflammatory mechanism that provides a protective effect on the gut against epithelial barrier dysfunction and alleviates inflammatory injury in intestine and remote organs. However, due to complicated manipulation and untoward side effects, including serious tissue injury, it is still difficult to apply electrical stimulation to the vagus nerve in clinical practice. Therefore, a more clinically desirable alternative therapy is needed during the resuscitative phase of trauma care. Acupuncture as one of the therapeutic maneuvers in traditional Chinese medicine (TCM) has been applied in clinics for thousands of years, and it has been found to have a bidirectional neuron-endocrine-immune system regulating effect, and antagonize the systemic inflammatory response without side effects. The authors demonstrated that electroacupuncture (EA) at Zusanli had a significant positive therapeutic effect in hemorrhagic shock rats with delayed fluid resuscitation, however, its mechanism remains unknown. The authors had also proved that EA can alleviate intestinal barrier insult and system inflammation in a rat ischemia model through activating the cholinergic anti-inflammatory pathway.

### Innovations and breakthroughs

The most important novel findings from this study are that when delayed resuscitation is inevitable during emergency situations such as hemorrhagic shock occurring in the battlefield or site of an unexpected accident or a disaster without sufficient fluids, EA at ST36 can be performed and it can successfully attenuate systemic inflammation, decrease gut injury and permeability and improve blood pressure and outcomes, which is consistent with preserved intestinal barrier function after hemorrhage and delayed fluid resuscitation.

### Applications

The study results provide evidences for acupuncture as an emergency intervention of anti-shock and protection of gut barrier function after severe hemorrhage and trauma.

### Terminology

Cholinergic anti-inflammatory pathway: The cholinergic anti-inflammatory pathway is a neural mechanism that inhibits the expression of cytokine through the interaction of the principle vagus nerve neurotransmitter, acetylcholine, and the  $\alpha 7nAChR$  subunit located on cytokine expressing cells by stimulating the vagus nerve by either electrical or pharmacological methods. EA: EA is a modification of conventional acupuncture that stimulates acupoints with electrical current instead of manual manipulations and appears to have more consistently reproducible results in both clinical and research settings.

### Peer review

The study is somewhat interesting, well designed and results seem to be very congruent among the different aspects of systemic inflammation and intestinal barrier function investigated.

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## Gastrointestinal stromal tumors of the duodenum: Surgical management and survival results

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### Abstract

**AIM:** To provide long-term survival results of operable duodenal gastrointestinal stromal tumors (DGISTs) in a tertiary center in China.

**METHODS:** In this retrospective study, the pathological data of 28 patients with DGISTs who had been treated surgically at the Second Department of General Surgery, Sir Run Run Shaw Hospital (SRRSH) from June 1998 to December 2006 were reviewed. All pathological slides were examined by a single pathologist to confirm the diagnosis. In patients whose diagnosis was not confirmed by immunohistochemistry at the time of resection, representative paraffin blocks were reassembled, and sections were studied using antibodies against CD117 (c-kit), CD34, smooth muscle actin (SMA), vimentin, S-100, actin (HHF35), and desmin. Operative procedures were classified as wedge resection (WR), local resection with pure closure, without duodenal transection or anastomosis), segmental resection [SR,

duodenal transection with Roux-Y or Billroth II gastrojejunostomy (G-J), end-to-end duodenoduodenostomy (D-D), end-to-end or end-to-side duodenojejunostomy (D-J)], and pancreaticoduodenectomy (PD, Whipple operation with pancreatojejunostomy). R0 resection was pursued in all cases, and at least R1 resection was achieved. Regional lymphadenectomy was not performed. Clinical manifestations, surgery, medical treatment and follow-up data were retrospectively analyzed. Related studies in the literature were reviewed.

**RESULTS:** There were 12 males and 16 females patients, with a median age of 53 years (20-76 years). Their major complaints were "gastrointestinal bleeding" (57.2%) and "nonspecific discomfort" (32.1%). About 14.3%, 60.7%, 17.9%, and 7.1% of the tumors originated in the first to fourth portion, respectively, with a median size of 5.8 cm (1.6-20 cm). Treatment was by WR in 5 cases (17.9%), SR in 13 cases (46.4%), and by PD in 10 cases (35.7%). The morbidity and mortality rates were 35.7% and 3.6%, respectively. The median post-operative stay was 14.5 d (5-47 d). During a follow-up of 61 (23-164) mo, the 2-year and 5-year relapse-free survival was 83.3% and 50%, respectively. Eighty-four related articles were reviewed.

**CONCLUSION:** Surgeons can choose to perform limited resection or PD for operable DGISTs if clear surgical margins are achieved. Comprehensive treatment is necessary.

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**Key words:** Gastrointestinal stromal tumors; Duodenum; Limited resection; Pancreaticoduodenectomy; Survival

**Core tip:** Duodenal gastrointestinal stromal tumors (DGISTs) represent a subset of small bowel gastrointestinal stromal tumors that require special consideration given their clinical manifestations, particularly difficult surgical

decisions and poor prognosis. Surgeons can choose to perform limited resection or pancreaticoduodenectomy for operable DGISTs according to the tumor size, location, proximity to the duodenal papilla, and their technical feasibility, and both these two approaches lead to a similar oncological prognosis if clear surgical margins are achieved. The prognosis of a DGIST is poor, thus comprehensive treatment is necessary.

Liang X, Yu H, Zhu LH, Wang XF, Cai XJ. Gastrointestinal stromal tumors of the duodenum: Surgical management and survival results. *World J Gastroenterol* 2013; 19(36): 6000-6010 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6000.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6000>

## INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract, although the annual incidence rates reported worldwide are less than 20 per million, and only about 5000 new cases are diagnosed annually in the United States<sup>[1-3]</sup>. GIST is a primary gastrointestinal disease that can arise anywhere along the digestive tract in adults. The stomach (60%) and jejunioileum (30%) are the most common primary sites, and only a small number of cases have been reported in the colorectum (< 5%), esophagus and appendix (< 1%)<sup>[4,5]</sup>. In addition, duodenal lesions represent approximately 5% of GISTs.

All GISTs harbor some malignant potential, although only 10%-30% are clinically malignant. In the past decade following Fletcher's report<sup>[6]</sup>, primary GISTs are not classified as "benign" or "malignant", but are stratified by the probability of recurrence after complete resection into very low, low, intermediate, and high risk on the basis of their size and mitotic rate. Subsequently, Miettinen *et al.*<sup>[7]</sup> suggested that the anatomical origin may be another independent factor for risk stratification, indicating that DGISTs share maximal risks with rectal GISTs compared with those of the stomach and jejunioileum.

Although DGISTs are relatively rare, they account for nearly 30% of all primary tumors of the duodenum, and the vast majority of patients present with gastrointestinal bleeding<sup>[4]</sup>. With regard to treatment, DGISTs often pose difficult surgical problems, due to the complex anatomical relationship around the duodenum, *i.e.*, unlike the stomach or other intestinal segments where complete excision with wide margins are relatively straightforward procedures, wide resection of DGISTs will almost always entail a pancreaticoduodenectomy (PD), which is massively invasive and technically challenging<sup>[8,9]</sup>.

In recent years, a limited resection (LR) of DGISTs demonstrated a comparable effect to PD in selected cases<sup>[10]</sup>. However, the optimal surgical approach (LR or PD) for DGISTs is largely unknown, as all the available evidence has been derived from small retrospective

series<sup>[11]</sup>. In addition, scholars have gradually recognized the complexities of DGISTs, and these tumors have been classified separately from other small intestine GISTs into an independent category<sup>[12]</sup>. Also, a number of papers on DGISTs have been released<sup>[8-10,13-16]</sup>. Nevertheless, more experience with long-term oncological observations is required, especially for surgeons. This article aims to provide a single center experience of operable DGIST cases in China, and an update on the clinical management of DGISTs.

## MATERIALS AND METHODS

### Data collection

In this retrospective study, the pathological data of 28 patients with DGISTs who had been treated surgically at the Second Department of General Surgery, Sir Run Run Shaw Hospital (SRRSH) from June 1998 to December 2006 were reviewed. All data were collected once a definite diagnosis had been made. The author (Xiu-Jun Cai) managed his first case of DGIST as an independent attending and maintained his interests. During this review period, the patients of five attending surgeons in our department were included, and the priority of these data was approved by the patients while in hospital and by the surgeons. In addition, the patients were confirmed as cases by an inverse retrieve from the inpatient system of our hospital. This study was approved by the Institutional Review Board of SRRS.

All pathological slides were reviewed by a single pathologist to confirm the diagnosis. In patients whose diagnosis was not confirmed by immunohistochemistry at the time of resection, representative paraffin blocks were reassembled, and sections were studied using antibodies against CD117 (c-kit), CD34, smooth muscle actin (SMA), vimentin, S-100, actin (HHF35), and desmin. Tumors were classified as GISTs only if tumor cells were characterized by the typical morphology with positive staining for CD117 and/or CD34. Patient age, gender, presentation, medical history, laboratory and radiology examinations, surgery, medical treatment and follow-up data were obtained from patient records, including operative notes, pathology reports, and outpatient data. None of the patients were lost to follow-up due to good communication between the authors, patients and their primary care providers.

Operative procedures were classified as wedge resection (WR, local resection with pure closure, without duodenal transection or anastomosis), segmental resection [SR, duodenal transection with Roux-Y or Billroth II gastrojejunostomy (G-J), end-to-end duodenoduodenostomy (D-D), end-to-end or end-to-side duodenojejunostomy (D-J)], and pancreaticoduodenectomy (PD, Whipple operation with pancreatojejunostomy). R0 resection was pursued in all cases, and at least R1 resection was achieved. Regional lymphadenectomy was not performed.

### Statistical analysis

The overall survival (OS), disease-related survival (DRS), and relapse-free survival (RFS) were conventionally de-

**Table 1** Summary of patient preoperative information

No.	Age (yr)	Sex	Chief complaint	Comorbidity	Past history	Hb	US	CT	MRI	GI	ES	EUS	DSA	Biopsy	Preoperative diagnosis
1	28	F	Melena	Cholecystolithiasis		46	0	1	N	1	1	N	N	1/ES	Duodenal GIST(Biopsy)
2	48	F	Incidentally found		Sub gastrectomy	79	0	1	N	1	N	N	N	N	Duodenal tumor
3	60	M	Melena			104	1	1	N	1	0	N	N	0/CT	Duodenal GIST (CT)
4	70	M	Pain			128	0	1	1	1	1	1	N	1/EUS	Duodenal GIST(Biopsy)
5	71	M	Incidentally found			130	0	0	N	N	0	N	N	N	Abdominal tumor
6	76	F	Melena	Cholecystolithiasis	Appendectomy, stripping of right great saphenous vein	65	0	1	N	N	1	1	N	0/EUS	Duodenal GIST (EUS)
7	42	F	Melena			50	0	1	N	N	N	N	N	N	Duodenal tumor
8	74	M	Melena			81	1	1	N	N	1	N	N	0/ES	Duodenal tumor
9	53	F	Melena			75	1	1	N	N	1	N	N	1/ES	Duodenal GIST(Biopsy)
10	47	F	Melena		Schistosomiasis	99	0	1	N	1	0	N	N	N	Duodenal tumor
11	55	F	Melena			61	1	1	N	1	0	N	N	N	Duodenal tumor
12	51	M	Melena			66	1	1	N	1	1	N	N	1/ES	Duodenal GIST (Biopsy)
13	50	M	Hematemesis	Polyp of gallbladder (1.9 cm)	Essential hypertension	52	0	0	N	N	1	N	N	0/ES	Duodenal tumor
14	69	F	Pain			87	0	0	N	N	N	N	N	N	Retroperitoneal tumor
15	65	M	Melena			65	1	1	N	N	1	1	N	0/EUS	Duodenal GIST (EUS)
16	63	M	Acute abdomen			148	1	N	N	N	N	N	N	N	Acute abdomen
17	44	F	Hematemesis			56	0	1	N	1	1	N	N	0/ES	Duodenal tumor
18	57	F	Discomfort		Cholecystectomy, left nephrectomy	126	1	1	N	N	N	N	N	N	Duodenal GIST (CT)
19	20	F	Melena			61	0	1	N	N	1	N	N	0/ES	Duodenal tumor
20	52	M	Pain			118	0	0	N	N	N	N	N	N	Abdominal tumor
21	53	F	Pain			118	0	0	N	1	N	N	N	N	Abdominal tumor
22	71	F	Early satiety			131	0	1	1	1	0	1	N	0/EUS	Retroperitoneal tumor
23	53	M	Early satiety		Resection of gluteal hemangioma	156	0	1	1	N	1	1	N	0/EUS	Duodenal GIST (EUS)
24	50	F	Melena	Cholecystolithiasis	Right radical mastectomy, hysteromyomectomy	91	0	1	N	N	1	N	N	0/ES	Duodenal tumor
25	46	F	Pain			93	0	0	N	N	N	N	N	N	Abdominal tumor
26	55	M	Melena		Appendectomy	69	0	0	N	N	1	N	N	0/ES	Tumor of pancreas head
27	51	M	Incidentally Found			155	0	1	1	0	0	N	N	N	Duodenal tumor
28	46	F	Melena		Radical cystectomy	87	0	1	N	N	N	N	1	N	Duodenal GIST (CT)

0: Negative; 1: Positive; N: Not evaluated; Hb: The initial hemoglobin (g/L) before liquid resuscitation; M: Male; F: Female; CT: Computed tomography; US: Ultrasonography; GIST: Gastrointestinal stromal tumor; MRI: Magnetic resonance imaging; GI: Gastrointestinal; ES: Gastroduodenoscopy; EUS: Endoscopic ultrasonography; DSA: Digital subtraction angiography.

fined. Survival was determined using the Kaplan-Meier method, and Cox regression was employed for multivariate analysis. A Pearson's, Spearman Rank, or Kendall's tau-b correlation was evaluated between variables if appropriate, and differences were analyzed using the Mann-Whitney *U* or unpaired Student's *t* test. All tests were two-sided and *P* values less than 0.05 were considered statistically significant. Software including Prism v5.04 (GraphPad Software Inc., La Jolla, CA, United States), SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, United States), and the Thomson Data Analyzer (Thomson Reuters Corp., New York, NY, United States) were used

for statistics and literature reviews.

## RESULTS

### Patients

There were 12 males and 16 females patients, aged  $54.3 \pm 2.4$  years (mean  $\pm$  SEM if Gaussian distributed, median: 53, range: 20-76 years), and male patients ( $50.3 \pm 3.6$  years) were younger than female patients ( $59.6 \pm 2.5$  years,  $P = 0.0595$ ). The chief complaint (lead symptom) was summarized as "gastrointestinal bleeding" (57.2%), "nonspecific discomfort" (32.1%), and "incidentally

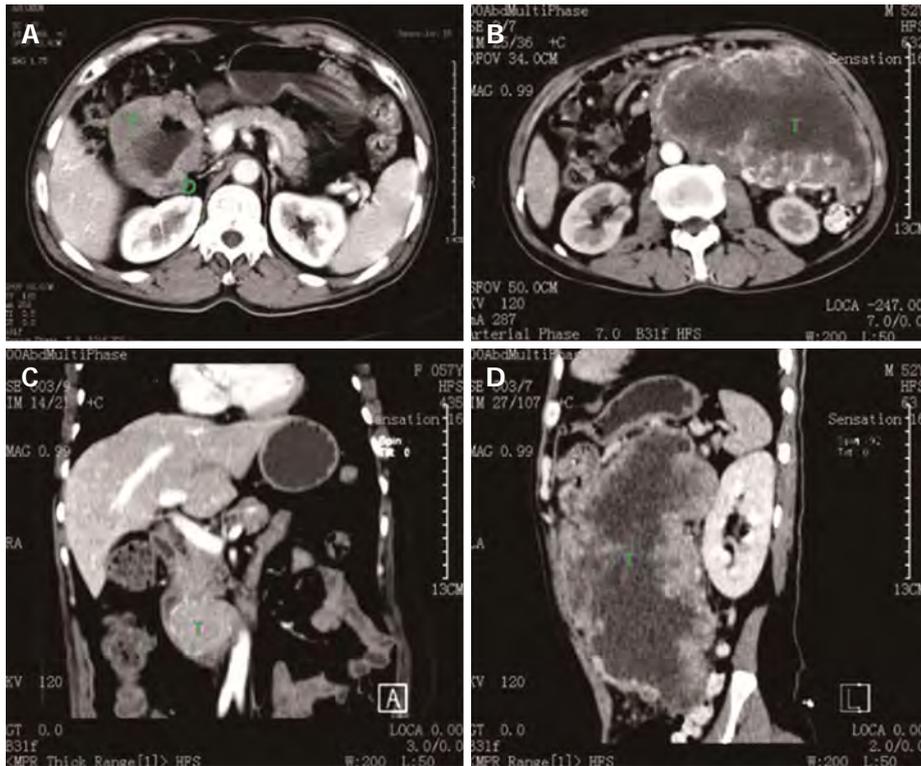


Figure 1 Respective computed tomography images. A: For case 28; B, D: For case 20; C: For case 18. (T: Tumor; D: Duodenum).

found" (10.7%); the symptoms were not correlated with tumor size or site ( $P > 0.05$ ). Four patients (14.3%) had comorbid gallbladder diseases. Seven patients (25.0%) had undergone one or two previous operations, two of which were for malignancy, and another two patients had essential hypertension or schistosomiasis. None of the patients had diabetes or neurofibromatosis (Table 1).

The hemoglobin in all patients was  $92.8 \pm 6.3$  g/L, which was highly correlated with the symptoms: the hemoglobin in patients with "gastrointestinal bleeding" was  $70.5 \pm 4.4$  g/L, whereas that of "non-gastrointestinal bleeding" patients ( $n = 12$ ) was  $122.4 \pm 7.3$  g/L ( $P < 0.0001$ ).

Each patient underwent abdominal ultrasonography (US), with 100% clinical availability, and only eight patients had positive results for DGISTs or duodenal tumors (vague reports of abdominal tumors were defined as negative), giving a sensitivity of 28.6% (8/28). The clinical availability of computed tomography (CT), magnetic resonance imaging (MRI), upper gastrointestinal barium examination (GI), gastroduodenoscopy (ES), endoscopic ultrasonography (EUS), digital subtraction angiography (DSA), and preoperative biopsy guided by CT, ES or EUS were 96.4%, 14.3%, 39.3%, 67.9%, 17.9%, 3.6%, and 53.6%, respectively. The sensitivities in sequence were 74.1%, 100%, 90.9%, 68.4%, 100%, 100%, and 26.7%. Collectively, twenty patients (71.4%) were diagnosed with duodenal tumors preoperatively, including ten (35.7%) with DGISTs; four patients (14.3%) were diagnosed with abdominal tumors of uncertain origin; minor diagnoses were retroperitoneal tumors (7.1%),

tumors of the pancreas head (3.6%) and acute abdomen (3.6%). Representative images are shown in Figures 1-3.

### Surgery

All surgical techniques were performed under general anesthesia. Intraoperatively, all tumors were single, solid, encapsulated but fragile, part of which had an irregular thick-walled necrotic core or multiple necrotic loculi. Four tumors originated in the bulb (D1, 14.3%), seventeen in the descending section (D2, 60.7%), five in the horizontal section (D3, 17.9%), and two in the ascending section (D4, 7.1%). The tumor size varied from 1.6 cm to 20 cm with a median of 5.8 cm (95%CI: 5.3-8.6), and was independent of the tumor site ( $P > 0.05$ ) (Table 2).

Five patients (17.9%) underwent a WR, 13 (46.4%) a SR, and 10 (35.7%) a PD. When SR patients were subdivided, four G-J (30.8%), four D-D (30.8%), and five D-J (38.4%) reconstructions were carried out. In addition, six concomitant operations were performed, *i.e.*, four cholecystectomies for gallbladder comorbidities and two intestinal resections for iatrogenic vessel injuries in the mesocolon transversum or the root of the small bowel mesentery.

Perioperative blood transfusions were common (78.6%) in this cohort, and eleven patients (39.3%) required intensive care as a postoperative transition, staying for 1-5 d (median: 2 d; 95%CI: 1.2-3.4 d). Eight (28.6%) major early complications occurred, including leakage of the choledochoenterostomy/duodenojejunostomy (7.1%) and delayed gastric emptying (DGE, 21.4%). Consequently, due to the intraoperative and early postop-

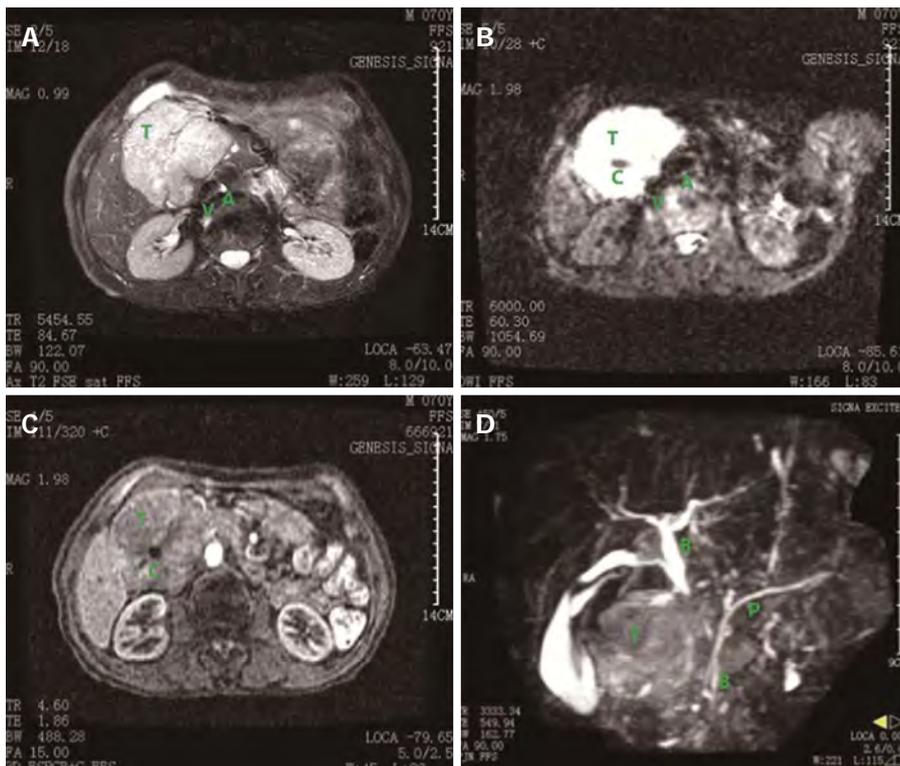


Figure 2 Respective magnetic resonance imaging images. A-C: For case 4; D: For case 22. T: Tumor; C: Necrotic core; A: Abdominal aorta; V: Inferior vena cava; B: Common bile duct; P: Main pancreatic duct.

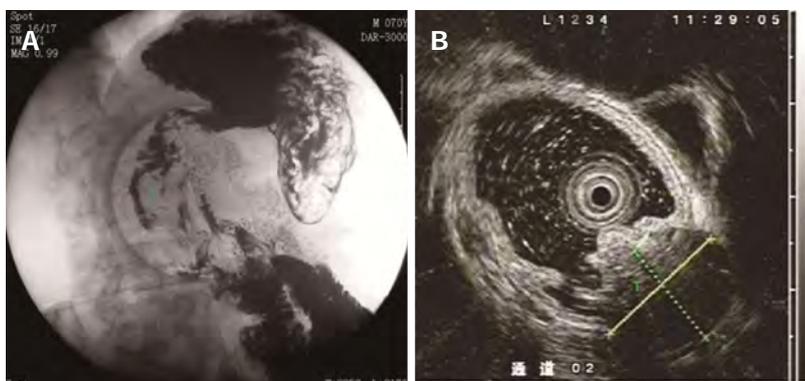


Figure 3 Respective gastrointestinal and endoscopic ultrasonography images. A: Gastrointestinal for case 4; B: Endoscopic ultrasonography for case 23. T: Tumor.

erative morbidities, the following three reoperations were performed: a total enterectomy for the mesenteric root injury; an abdominal irrigation and drainage for the choledochoenterostomy failure; and a gastrojejunostomy for DGE.

The overall post-operative stay was 5-47 d (median: 14.5 d; 95%CI: 14.2-24.1 d), and was closely correlated with the surgical approaches; the intervals from the WR, PD, and SR to discharge were  $10.6 \pm 1.3$ ,  $14.6 \pm 2.5$  ( $P > 0.05$  for WR), and  $25.9 \pm 4.1$  d ( $P = 0.0412$  for PD,  $P = 0.0393$  for WR), respectively.

### Pathology and risk classifications

The positive rates of the four principal immunohisto-

chemistry markers, CD117, CD34, SMA, and S-100 were 96.4%, 64.3%, 60.7%, and 42.9%, respectively. Moreover, another three markers were introduced in certain patients: vimentin (13/13, total/positive), actin (18/8), and desmin (9/0). According to Fletcher's criterion 6, there were two patients (7.1%) with very low risk, nine patients (32.1%) with low risk, six patients (21.4%) with intermediate risk, and 11 patients (39.3%) with high risk, however, by applying Miettinen's criterion 7, 11 patients (39.3%) showed low risk and the other 17 (60.7%) possessed high risk (Table 3).

### Survival analysis

The median OS was 64.5 mo, including three elderly pa-

Table 2 Summary of surgery and perioperative information

No.	Surgery	Site	Size (L)	Size (W)	Size (H)	Combined operation	Transfusion <sup>1</sup>	ICU stay	Major complications	Reoperation (post-operative time)	POS
1	SR (G-J)	D2	4	3.5	3	Cholecystectomy	Y	0		Hepatectomy for liver metastasis (103 mo)	22
2	WR	D2	4	3	3		Y	2			12
3	PD	D2	10	9	7.5		Y	0			17
4	PD	D2	11	9	7.5		Y	1	Leakage of choledochointerostomy	Abdominal irrigation and drainage (8 d)	14
5	SR (D-D)	D3	9	6	6	Partial colectomy	N	1	vessel injury in the mesocolon transversum		11
6	SR (D-J)	D2	4	4	4	Cholecystectomy	Y	5	Anastomotic leakage		36
7	PD	D2	6	5.5	5		Y	0			6
8	WR	D1	3.5	2.5	2.5		Y	1			8
9	SR (D-D)	D1	2.5	1.5	1.5		Y	0			5
10	PD	D2	4	3	2.5		Y	0			11
11	SR (G-J)	D4	8	5.5	3		Y	0			10
12	PD	D2	12	10	8.5		Y	0			10
13	WR	D2	3.5	3.2	3	Cholecystectomy	Y	0			15
14	PD	D3	13	11	11	Major enterectomy	Y	5	Injury of superior mesenteric vessels	Total enterectomy (1 d)	5
15	SR (D-J)	D3	5	4	3.5		Y	0	DGE	Gastrojejunostomy (21 d)	32
16	PD	D2	6.5	5	4		N	4			24
17	PD	D2	9.5	8.5	8		Y	1		Gastroscopic injection of sclerosing agents (21 mo)	28
18	SR (D-J)	D3	5.5	5	4		N	0	DGE		46
19	SR (D-D)	D2	2	2	2		Y	0	DGE		47
20	SR (D-J)	D4	20	18	8.5		Y	2	DGE		26
21	WR	D2	15	12	6.5		Y	0			10
22	SR (G-J)	D2	5.5	5	2.5		Y	2			42
23	WR	D1	1.6	1.3	1.2		N	0			8
24	SR (G-J)	D1	4.5	3	2	Cholecystectomy	N	0	DGE		17
25	SR (D-D)	D2	7.6	5	4.6		Y	0			8
26	PD	D2	6	4	4		Y	0		Adhesiolysis for ileus (43 mo)	9
27	SR (D-J)	D3	5.5	4.5	3.5		N	1	DGE		35
28	PD	D2	6	5	4		Y	0			22

<sup>1</sup>Perioperative transfusion, including the transfusion before surgery; Size (L/W/H): tumor size (length, width and height in centimeters) measured after fixation by 10% neutral buffered formalin; DGE: Delayed gastric empty; POS: Post-operative stay till discharge; ICU: Intensive care unit; PD: Pancreaticoduodenectomy; WR: Wedge resection; SR: Segmental resection.

tients who died of lung cancer, pneumonia, and stroke, respectively. The median RFS and DRS in the whole patient group and subgroups are listed in Table 4, and the results of multivariate analysis are shown in Table 5. Moreover, a nomogram developed by Gold *et al*<sup>[17]</sup> to predict the probability of 2- and 5-year RFS was used, and the predicted values were compared to the actual values (Table 4 and Figure 4).

## DISCUSSION

GISTs are a family of tumors thought to arise from the interstitial cells of Cajal in the gastrointestinal tract. Recently, the putative stem and progenitor cells for GISTs have been identified<sup>[1]</sup>. Most GISTs have oncogenic mutations in either KIT or platelet-derived growth factor receptor- $\alpha$  (PDGFRA), and there is substantial evidence that these mutations are pathogenetic for the initiation of GISTs. Histopathologically, GISTs are usually well circumscribed and surrounded by a pseudocapsule, ranging in size from millimeters to 40 cm, with a median size between 5 cm and 8 cm, while large GISTs often show

cystic degeneration or central necrosis<sup>[18]</sup>. Microscopically, GISTs are defined as morphologically spindle cell, epithelioid, or occasionally pleomorphic, mesenchymal tumors, usually (approximately 95%) express the KIT protein and often (up to 90%) harbor mutations of a gene that encodes for a type III receptor tyrosine kinase (either KIT or PDGFRA)<sup>[19]</sup>.

Surgery is the mainstay of treatment for localized, resectable GISTs. The tumor should be removed *en-bloc* with its pseudocapsule to yield an adequate resection margin. The optimal width of the tumor-free margin has not been defined, and it is unclear if re-resection is beneficial for positive microscopic surgical margins (R1), especially as the free radial margin is the one that is positive in most instances and there is no additional tissue to be removed<sup>[20]</sup>. Lymphadenectomy is not warranted unless there is gross nodal involvement. In cases of unresectable or marginally resectable disease, adjuvant tyrosine kinase inhibitor (TKI) therapy should be considered. Following surgical resection, GISTs often recur locally, spread diffusely throughout the serosal surfaces of the abdomen and/or metastasize to the liver. Advanced disease is associated with

**Table 3 Summary of pathological data and risk classifications**

No.	CD117	CD34	SMA	S100	Vimentin	Actin	Desmin	Mitotic rate/50HPF	Fletcher's risk	Miettinen's risk	UICC TNM	Gold's point	RFS	DRS	Status of death	Glivec
1	1	0	1	0	N	0	0	0-1	L	L	UICC I	64	103	164	0	0
2	1	1	1	0	1	0	N	0-1	L	L	UICC I	64	RF	146	0	0
3	1	0	1	0	1	0	N	5-8	H	H	UICC III B	173	54	61	1	0
4	1	1	1	1	1	0	N	>10	H	H	UICC III B	175	26	35	1	0
5	1	1	1	0	1	N	N	1-2	I	H	UICC II	89	RF	23	1 (lung cancer)	0
6	1	1	0	1	1	1	0	2-3	L	L	UICC I	64	RF	25	1 (pneumonia)	0
7	1	0	1	1	1	N	0	6-8	H	H	UICC III B	155	64	73	1	0
8	0	1	0	0	1	1	N	0-1	L	L	UICC I	61	RF	61	1 (stroke)	0
9	1	1	1	1	N	0	N	3-4	L	L	UICC I	55	RF	116	0	0
10	1	0	1	1	N	0	N	0-1	L	L	UICC I	64	101	111	1	0
11	1	1	0	1	N	0	N	5-6	H	H	UICC III B	164	29	55	1	0
12	1	1	0	1	1	N	N	>10	H	H	UICC III B	178	15	23	1	0
13	1	1	1	0	1	0	N	0-1	L	L	UICC I	61	RF	102	0	0
14	1	1	1	0	1	1	N	>10	H	H	UICC III B	180	NN	NN	1	0
15	1	1	1	0	N	0	N	1-4	L	L	UICC I	70	62	68	1	0
16	1	1	0	1	1	0	N	5-8	H	H	UICC III B	156	37	49	1	0
17	1	1	0	1	N	0	N	>10	H	H	UICC III B	172	21	33	1	0
18	1	1	0	0	N	0	N	2-3	I	H	UICC II	71	53	57	1	0
19	1	0	1	0	N	N	0	0-1	VL	L	UICC I	51	RF	86	0	0
20	1	1	0	0	N	N	0	>10	H	H	UICC III B	190	22	33	1	0
21	1	0	1	0	N	0	N	5-8	H	H	UICC III B	184	29	40	1	0
22	1	1	0	0	N	0	0	1-2	I	H	UICC II	71	47	59	1	0
23	1	0	1	1	N	N	0	0-1	VL	L	UICC I	50	RF	75	0	0
24	1	0	1	0	N	N	0	3-5	L	L	UICC I	67	69	73	0	0
25	1	0	1	0	N	0	N	0-1	I	H	UICC II	81	51	71	0	1 (20 mo/PR)
26	1	0	0	1	1	N	N	5-7	H	H	UICC III B	155	59	70	0	0
27	1	1	0	0	N	N	0	0-1	I	H	UICC II	71	RF	63	0	1 (24 mo)
28	1	1	1	1	1	N	N	0-1	I	H	UICC II	73	RF	61	0	0

0: Negative; 1: Positive; N: Not evaluated; HPF: High-power fields; VL: Very low; L: Low; I: Intermediate; H: High; RF: Relapse free; NN: Not necessary; PR: Partial remission; UICC: Union for International Cancer Control; TNM: Tumor, nodes, metastasis; RFS: Relapse-free survival; DRS: Disease-related survival; SMA: Smooth muscle actin.

**Table 4 Results of survival analysis**

	n	Median RFS (mo)	95%CI of HR	Median DRS (mo)	95%CI of HR	Median Gold's point (range)	Predicted 2-year RFS probability	Actual 2-year RFS rate	Predicted 5-year RFS probability	Actual 5-year RFS rate
Total	25	60.5	-	73	-	73 (50-190)	83%	83.30%	70%	50%
Fletcher's risk										
Very low/low	9	103 <sup>c,e</sup>	1.564-158.6	Undefined	0.6931-149.1	64 (50-70) <sup>d,f</sup>	86%	100%	75%	100%
Intermediate	5	53	0.7675-7.845	Undefined	0.9385-10.78	71 (71-81) <sup>b,f</sup>	84%	100%	70%	40%
High	11	29 <sup>b,d</sup>	4.812-68.51	40 <sup>b,d</sup>	3.449-44.94	173 (155-190) <sup>b,d</sup>	<10%	70%	<10%	<10%
Miettinen's risk										
Low	9	103 <sup>f</sup>	2.882-26.67	Undefined	2.224-21.94	64 (50-70) <sup>f</sup>	86%	100%	75%	100%
High	16	47 <sup>b</sup>	-	56	-	160 (71-190) <sup>b</sup>	<10%	80%	<10%	20%

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs Very low/Low; <sup>c</sup>P < 0.05, <sup>d</sup>P < 0.01 vs Intermediate; <sup>e</sup>P < 0.05, <sup>f</sup>P < 0.01 vs High; DRS: Disease-related survival; RFS: Relapse-free survival.

metastases to distant sites, including the lung and bone. Prior to the advent of TKI therapeutics, the prognosis for advanced GISTs was poor owing to their inherent resistance to both chemotherapy and radiation therapy<sup>[1]</sup>.

DGISTs share the above-mentioned factors, but have individuality. DGISTs are unique entities, not only due to their anatomical location, but also their clinical manifestations, particularly difficult surgical decisions and poor prognosis. This is why DGISTs have attracted the authors' interests as well as the attention of the French Sarcoma Group (GSF-GETO). In the 47<sup>th</sup> Annual Meet-

ing of the American Society of Clinical Oncology held in Chicago in June 2011, Duffaud *et al*<sup>[12]</sup> at GSF-GETO12 retrospectively analyzed 66 resectable DGIST patients with a median tumor size of 6 cm (1.5-31 cm), 29 of whom underwent WR, 23 SR, and 14 PD. During a median follow-up of 36 (1-168) mo, their 4-year OS and RFS rates were 89% and 58%, respectively. Duffaud's report is the largest cohort study in the surgical rather than pathological field, thus has current significance.

The clinical presentations of DGISTs are highly variable according to their size and the existence of mucosal

Table 5 Results of Cox multivariate analysis		
Variable	RFS <i>P</i> value	DRS <i>P</i> value
Mitotic rate/50HPF	0.0000	0.0000
Gold's point	0.0000	0.0000
Fletcher's risk	0.0000	0.0000
Miettinen's risk	0.0001	0.0009
Size > 5 cm	0.0001	0.0009
Preoperative diagnoses as duodenal tumors or DGISTs	0.0048	> 0.05
Age > 60 yr	0.0428	0.0059
LR or PD	> 0.05	0.0346
ICU stay	> 0.05	0.0054

HPF: High-power fields; DRS: Disease-related survival; RFS: Relapse-free survival; ICU: Intensive care unit; LR: Limited resection; PD: Pancreaticoduodenectomy.

ulceration, but not tumor site<sup>[8,9]</sup>. The most common clinical presentation is reported to be gastrointestinal bleeding or abdominal pain. Interestingly in this series, the authors found that large tumors (> 5 cm) caused less gastrointestinal bleeding ( $P < 0.05$ ); this can be rationalized by the different phenotype of DGISTs, “submucosal/ulcerous type” or “serosal/massive type”, most small tumors were the former type and the majority of large tumors were the latter type<sup>[21]</sup>. More than 60% of DGISTs are located in the descending section, however, the reason for this is unclear.

Preoperatively, a variety of alternative examinations can be adopted, among which CT and MRI seem to be the best imaging modalities for assessment of the primary lesion and detection of metastases, whereas EUS is the optimum non-invasive tool for the clinical diagnosis<sup>[22,23]</sup>. Furthermore, EUS-guided biopsy has been established for the pathological diagnosis, although the sensitivity of DGIST samples obtained by EUS-guided biopsy is unsatisfactory compared with stomach GISTs (37.5% *vs* 84.4%)<sup>[24]</sup>. Recently, CT- or US-guided biopsy has been abandoned for resectable GISTs, due to the risk of pseudocapsule rupture and tumor spillage in the peritoneal cavity<sup>[4]</sup>. By integrating all the diagnostics, 71.4% of patients were accurately or probably diagnosed as having a DGIST. Moreover, the sensibility of preoperative diagnosis was correlated to Fletcher's or Miettinen's risk ( $P < 0.01$ ) and initial hemoglobin levels ( $P < 0.05$ ); that is, the tumor with malignant behavior has a tendency to be diagnosed as an abdominal/retroperitoneal tumor or a pancreatic cancer rather than a DGIST, whereas decreased hemoglobin potentially raises suspicion of gastrointestinal diseases, and possibly DGISTs.

The optimal surgical approach (LR or PD) for DGISTs is controversial. Goh *et al*<sup>[10]</sup> suggested that LR is associated with a shorter operation time, a similar complication rate, and a comparable disease-specific survival; Duffaud *et al*<sup>[12]</sup> concluded that LR rather than PD should be pursued to preserve optimal pancreas function for a better quality of life. According to the relatively few patients with long-term follow-up in this report, the findings support the views above. Although the DRS fol-

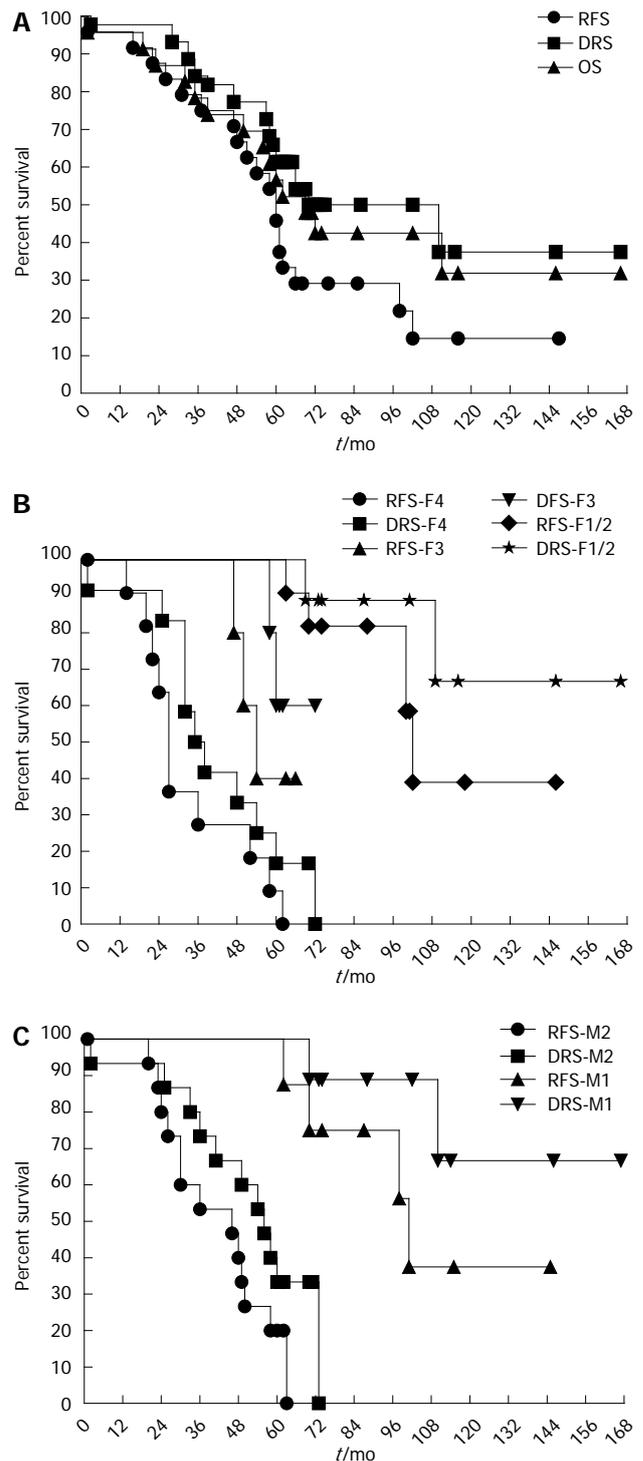


Figure 4 Survival curves. DRS: Disease-related survival; RFS: Relapse-free survival; OR: Overall survival; DFS: Disease free survival.

lowing PD seemed poor ( $P < 0.05$ ), after adjusting the covariates, PD tended to be performed in patients with high risk ( $P < 0.05$ ), and the results proved that LR and PD had a similar impact on RFS and DRS ( $P > 0.05$ ), thus, both surgical approach lead to a similar oncological prognosis if clear surgical margins are achieved<sup>[4,14]</sup>. Patients undergoing LR and PD showed similar overall morbidities (44.4% *vs* 20.0%;  $P > 0.05$ ) with the excep-

tion of DGE which was more frequent in the SR group (46.2%), and prolonged postoperative stay ( $P > 0.05$ ).

Regardless of the pros and cons outlined above, essential factors which influence whether LR or PD is chosen are tumor size, location, proximity to the duodenal papilla, and technical feasibility<sup>[25-28]</sup>. In general, WR with primary closure can be performed for small lesions if the resulting lumen is adequate and the Vater ampulla can be preserved, even by laparoscopy or combined laparoscopic surgery<sup>[29,30]</sup>. Occasionally, the antimesenteric defect after WR can be closed by Roux-en-Y duodenojejunostomy, whereas the mesenteric defect inside the “C” loop of the pancreas head can be repaired by translocation of the distal common bile duct as a patch<sup>[31,32]</sup>. SR with gastrojejunostomy or end-to-side/-end duodenojejunostomy may be performed for larger tumors located in the D1, D3 and D4. The side[D]-to-end[J] duodenojejunostomy is not recommended due to possible duodenal leakage and stump stasis (stump syndrome)<sup>[33]</sup>. Some scholars advocate resection and anastomosis even for lesions close to the papilla by performing the anastomosis just below the ampulla, which has been achieved by performing a lateromedial anastomosis opposite the papilla or by performing papilloplasty with a temporary stent catheter inserted into the papilla to avoid possible postoperative stenosis<sup>[34,35]</sup>. PD is only indicated when the tumor is located in the D2 and involves the papilla, pancreas, or if the tumor is large with high malignant potential and has involved the adjoining organs<sup>[8]</sup>. PD combined with major hepatectomy for a DGIST with localized liver metastases has also been reported<sup>[36]</sup>. PD can provide a wider tumor clearance, but reconstruction is difficult and there is an increased risk of long-term anastomotic stenosis, as both the pancreatic and common bile ducts are likely to be smaller in diameter<sup>[37]</sup>. In this context, the author introduced the binding pancreaticojejunostomy, which resolved these problems, improved the anastomotic operability, and decreased postoperative complications<sup>[38,39]</sup>. In addition, for ES-accessible mini tumors (less than 1.2 cm) in the D1, D2, and proximal D3, EUS-assisted band ligation is also feasible, although the necessity is debated<sup>[40]</sup>.

Beside complete resection, pharmacological treatments are necessary<sup>[41]</sup>. Glivec® (imatinib), a TKI, is now widely prescribed in the United States and Western countries for high-risk local GIST patients as adjuvant therapy after surgery and in metastatic GIST patients as first-line treatment<sup>[2,20,42]</sup>. However, in China, only 8.1% of urban patients (Table 3, 7.1% of present cases) take imatinib, although the Glivec® International Patient Assistance Program (GIPAP) was officially started in September 2003 by the China Charity Foundation, the Tumor Drug Department of Novartis, and the Max Foundation, with the objective of providing free medicines to patients who needed treatment. GIPAP has relatively strict eligibility criteria, and the annual Glivec® cost is far beyond the economic realities in China, therefore only a few urban patients benefit from this donation program, let alone

rural populations. Accordingly, neoadjuvant imatinib therapy is impractical in China, despite the fact that it is believed to allow LR in patients with locally advanced DGISTs<sup>[12]</sup>.

In terms of the survival analyses, Duffaud *et al*<sup>[12]</sup> indicated that only mitotic rate predicted RFS; whereas the present study showed that not only mitotic rate, but also tumor size and various combinations of these two parameters in addition to Gold's point, Fletcher's risk and Miettinen's risk predict RFS with similar statistical powers ( $P < 0.001$ ). Moreover, preoperative diagnosis of a duodenal tumor is also a positive factor for better patient survival, however, it is not an independent parameter. Gold *et al*<sup>[17]</sup> of the Memorial Sloan-Kettering Cancer Center have developed a nomogram to predict the probability of 2- and 5-year RFS for resected GISTs patients, and tested it in patients from the Spanish Group for Research on Sarcomas and the Mayo Clinic. With regard to the present cohort, Table 4 shows that the actual 2-year RFS rate was similar to the predicted value (83.3% *vs* 83%), but the actual 5-year RFS rate was lower than the predicted value (50% *vs* 70%), this may be due to the more malignant behavior of DGISTs compared with other small intestinal GISTs.

The limitations of this study include its retrospective design, small sample size, single center experience, and lack of adjuvant therapy. As analyses with small numbers of patients sometimes give misleading results, readers should be careful in evaluating these findings. However, based on a comprehensive literature review, it is necessary to strengthen these results. Future prospective studies enrolling larger numbers of patients and/or multiple medical centers are required.

## COMMENTS

### Background

Duodenal gastrointestinal stromal tumors (DGISTs) are a rare entity of gastrointestinal stromal tumors (GISTs), with characteristic clinical manifestations. Few cohorts with the exception of case studies have been reported. The purpose of this report is to provide long-term survival results of operable DGISTs in a tertiary center in China.

### Research frontiers

Although DGISTs are relatively rare, they account for nearly 30% of all primary tumors of the duodenum, and the vast majority present with gastrointestinal bleeding. With regard to treatment, DGISTs often pose difficult surgical problems, due to their complex anatomical relationship around the duodenum, *i.e.*, unlike the stomach or other intestinal segments where complete excision with wide margins are relatively straightforward procedures, wide resection of DGISTs will almost always entail a pancreaticoduodenectomy (PD), which is massively invasive and technically challenging.

### Innovations and breakthroughs

In recent years, a limited resection (LR) of DGISTs demonstrated a comparable outcome to PD in selected cases. However, the optimal surgical approach (LR or PD) for DGISTs is largely unknown, as all the available evidence has been derived from small retrospective series. In addition, scholars have gradually recognized the complexities of DGISTs, and these tumors have been classified separately from other small intestine GISTs into an independent category. Also, a number of papers on DGISTs have been released. Nevertheless, more experiences with long-term oncological observations are required, especially for surgeons.

### Applications

This article provides a single center experience of operable cases in China, and

an update on the clinical management of DGISTs.

### Terminology

GIST is the most common mesenchymal neoplasm of the gastrointestinal tract. GIST is a primary gastrointestinal disease that can arise anywhere along the digestive tract in adults.

### Peer review

DGIST represents a subset of small bowel GISTs that requires special consideration given its clinical manifestations, especially for the difficult surgical decisions and poor prognoses. Surgeons can choose the limited resection or pancreaticoduodenectomy for operable DGISTs according to the tumor size, location, proximity to the duodenal papilla, and their technical feasibility, and either of the two approaches leads to an indistinctive oncological prognosis as long as clear surgical margins are achieved. The prognoses of DGISTs are poor, thus a comprehensive treatment is necessary. The authors provided a single center experience of operable cases in China, and reviewed update on the clinical management of DGISTs.

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## Radiofrequency ablation for early oesophageal squamous neoplasia: Outcomes form United Kingdom registry

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### Abstract

**AIM:** To report outcomes on patients undergoing radiofrequency ablation (RFA) for early oesophageal squamous neoplasia from a National Registry.

**METHODS:** A Prospective cohort study from 8 tertiary referral centres in the United Kingdom. Patients with squamous high grade dysplasia (HGD) and early squamous cell carcinoma (ESCC) confined to the mucosa were treated. Visible lesions were removed by endoscopic mucosal resection (EMR) before RFA. Following initial RFA treatment, patients were followed up 3 monthly. Residual flat dysplasia was treated with RFA until complete reversal dysplasia (CR-D) was achieved or progression to invasive Squamous cell cancer defined as infiltration into the submucosa layer or beyond. The main outcome measures were CR-D at 12 mo from start of treatment, long term durability, progression to cancer and adverse events.

**RESULTS:** Twenty patients with squamous HGD/ESCC completed treatment protocol. Five patients (25%) had EMR before starting RFA treatment. CR-D was 50% at 12 mo with a median of 1 RFA treatment, mean 1.5 (range 1-3). Two further patients achieved CR-D with repeat RFA after this time. Eighty per cent with CR-D remain dysplasia free at latest biopsy, with median follow up 24 mo (IQR 17-54). Six of 20 patients (30%) progressed to invasive cancer at 1 year. Four patients (20%) required endoscopic dilatations for symptomatic structuring after treatment. Two of these patients have required serial dilatations thereafter for symptomatic dysphagia with a median of 4 dilatations per patient. The other 2 patients required only a single dilatation to achieve an adequate symptomatic response. One patient developed cancer during follow up after end of treatment protocol.

**CONCLUSION:** The role of RFA in these patients re-

mains unclear. In our series 50% patients responded at 12 mo. These figures are lower than limited published data.

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**Key words:** Squamous neoplasia; Oesophageal cancer; Endoscopic mucosal resection; High-grade dysplasia; Radiofrequency ablation

**Core tip:** Squamous cell cancer of the esophagus is an aggressive pathology with a poor prognosis and therefore early intervention is paramount to improve survival. Minimally invasive endoscopic therapy with endoscopic resection and radiofrequency ablation in patients with early squamous neoplasia of the esophagus has the potential to treat selected patients. Early response to RFA is prognostically important. Further studies with more patient numbers are required to explore the long and short term efficacy of this intervention in these complex patients.

Haidry RJ, Butt MA, Dunn J, Banks M, Gupta A, Smart H, Bhandari P, Smith LA, Willert R, Fullarton G, John M, Di Pietro M, Penman I, Novelli M, Lovat LB. Radiofrequency ablation for early oesophageal squamous neoplasia: Outcomes from United Kingdom registry. *World J Gastroenterol* 2013; 19(36): 6011-6019 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6011.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6011>

## INTRODUCTION

Squamous cell cancer (SCC) comprises nearly 90% of all esophageal cancers worldwide<sup>[1]</sup>. The incidence of esophageal SCC has fallen in the western world in the past 3 decades but still remains between 4 and 16 per 100000 population. This is strongly dependent on geographical location worldwide with figures far higher in Asia<sup>[2]</sup>. In the western world factors such as alcohol and tobacco play an important role in the development of oesophageal SCC<sup>[3]</sup>. This condition carries a poor prognosis with an overall five year survival rate of 10%-15%<sup>[4]</sup>. Those treated with surgery following neo-adjuvant therapy still carry a poor prognosis with a 5 year overall survival of about 33%. Surgery carries a significant mortality of 5% and postoperative morbidity of up to 47%<sup>[5]</sup>. The precursor lesion to SCC is known as squamous dysplasia. The World Health Organization (WHO) refers to squamous dysplasia as squamous intra-epithelial neoplasia and has further categorized the condition depending on the grade of dysplasia as low grade intra-epithelial neoplasia (LGIN) through to high grade intra-epithelial neoplasia (HGIN)<sup>[6]</sup>. Non invasive SCC is often referred to as ESCC.

Squamous high-grade dysplasia and ESCC carry a risk of progression to invasive SCC of up to 65% at 3.5 years and as high as 74% at 13.5 years<sup>[7]</sup>. The chance of lymph

node metastasis is dependent upon the penetration and depth of the lesion. Lesions restricted to the epithelial layer (mL) or the lamina propria (m<sup>2</sup>) have a low rate of lymph node metastases (< 5%); lesions that penetrate into the muscularis mucosae (m3) or the first third of the submucosa (sm1) have a higher risk (5%-15%)<sup>[8-12]</sup>. Once there is deeper submucosal (sm2 and sm3) involvement the risk of lymph node spread can be in the region of 24%<sup>[13,14]</sup> and surgical or oncological interventions are the treatments of choice. Traditional treatment for squamous neoplasia has been surgery or chemo-radiotherapy. However with disease limited to the mucosa the risk of lymph node involvement is low and minimally invasive endoscopic therapy is an alternative.

With the advances in minimally invasive esophageal endotherapy over the past decade there are now additional treatment options for patients with squamous HGD and ESCC confined to the lamina propria. Centers in Asia and the Western countries have shown that the use of EMR is effective and curative for these patients<sup>[15,16]</sup>. However this form of endotherapy is associated with significant oesophageal stenosis<sup>[17]</sup> and therefore alternative treatments have been desirable. The use of photodynamic therapy in this cohort of patients has again shown promising results<sup>[18]</sup> but is not widely available and again is associated with significant stenosis post treatment.

Endoscopic submucosal dissection (ESD) has been developed in Asia as one of the standard endoscopic resection techniques for early squamous neoplasia of the oesophagus. ESD enables oesophageal lesions, regardless of their size, to be removed en bloc and thus has a lower local recurrence rate than EMR. The *en bloc* resection rate is greater than 90% (90.6%-100%)<sup>[19-25]</sup>. *En bloc* resection, meaning resection in a single piece, facilitates an accurate histological assessment and reduces the risk of recurrence. In fact, the local recurrence rate after oesophageal ESD is extremely low (0%-3.1%)<sup>[21-25]</sup>. ESD is yet to become established in the united kingdom as this techniques is not widely available and is confined to specialist center's only.

RFA using the HALO system (BÂRRX Medical, Sunnyvale, California, United States) is a novel minimally invasive field ablation technique which has established efficacy for treating HGD and early adenocarcinoma arising in Barrett's esophagus (BE)<sup>[26]</sup>. The HALO System uses ultra short pulsed radiofrequency that ablates the mucosa whilst preserving the submucosa. Emerging evidence suggests that RFA is safe and efficacious in the management of squamous HGD<sup>[26-29]</sup>. We report the United Kingdom HALO registry experience of the first 20 patients with squamous HGD and ESCC who have completed treatment protocol.

The UK HALO RFA registry was created to audit outcomes of patients undergoing RFA for HGD/early neoplasia in BE and patients with squamous esophageal HGD/ESCC. It is a prospective multicenter registry which holds patient data from 20 centers nationwide.

## MATERIALS AND METHODS

### Ethical approval

Ethical approval was granted by the Joint UCL/UCLH Committee on the ethics of Human research (REC REF 08/H0714/27). The HALO ablation system has already been approved by the US Food and Drug Administration (FDA). In addition it is a European Cleared (CE) device as well as having been approved by the National Institute of Clinical Excellence (NICE) in the United Kingdom for treatment of HGD in BE.

### Inclusion criteria

All patients referred for consideration for endotherapy for squamous HGD and early SCC were invited to enter at collaborating centers. Patients had an endoscopic and histological diagnosis of squamous HGD or ESCC confirmed by two independent expert gastrointestinal histopathologist prior to embarking on endotherapy.

### Enrolment

Between January 2008 and March 2013 a total of 670 patients were enrolled to the registry from a total of 20 centers nationwide in the United Kingdom. Amongst these, 27 patients from 8 centers had squamous HGD or ESCC. Twenty of these have completed treatment protocol.

### Pre-enrolment staging

All patients were referred by the cancer center specialist multidisciplinary team (sMDT). All had endoscopic assessment with multiple biopsies to exclude invasive disease. All investigators used the Paris Classification to classify macroscopic lesions<sup>[30]</sup>. Enhanced endoscopic techniques including chromoendoscopy with Lugol's iodine, narrow band imaging (Olympus, Hamburg, Germany) and I-scan (Pentax, Hoya Corporation, Japan) were used to target areas of suspected squamous dysplasia (see Figure 1) depending on which technology was present in the respective hospitals. Endoscopic ultrasound (EUS) and CT scanning was performed according to sMDT requirements. Endoscopic ultrasound was used if there were visible lesions seen to ensure that the neoplasia was confined to the mucosa. Endoscopic mucosal resection (EMR) was performed for any raised visible lesions and assessed by two expert gastrointestinal histopathologists at each center. Four quadrant biopsies were then obtained every 2 cm through the oesophagus to map for any further neoplasia. If invasive cancer was detected, the patient was referred back to the sMDT for alternative therapy. Invasive cancer was defined as neoplasia invading into the submucosa such that it was no longer amenable to endoscopic therapy with EMR or RFA.

### Registry endoscopy protocol

Once consented patients had their first ablation as described below. At 3 mo all patients returned for follow up endoscopy where mapping biopsies were taken using chromoendoscopy with Lugol's iodine and enhanced endoscopic imaging. As well as targeted biopsies of USLs

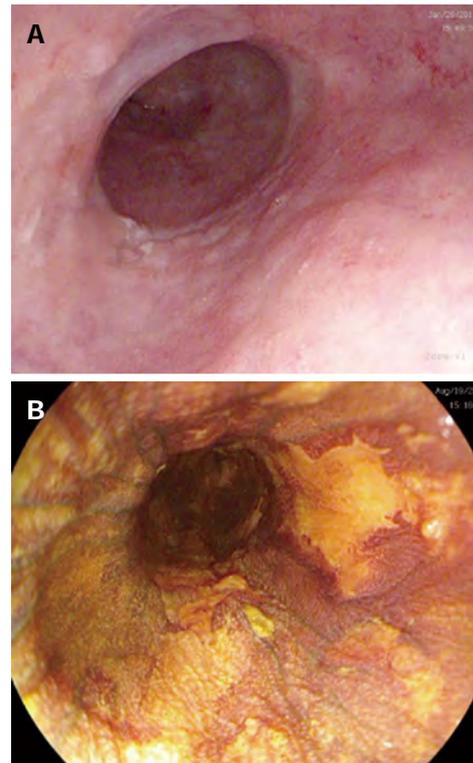


Figure 1 Endoscopic views of a segment of squamous high grade dysplasia with macroscopic lesions suggestive of dysplasia with (A) high definition white light endoscopy and confirmation of areas of dysplasia with (B) Lugol's iodine dye spray representing unstained lesions.

seen after Lugol's staining, systematic 4 quadrant biopsies every 2 cm were taken through the squamous esophagus to map any residual dysplasia. Any new raised visible lesions were treated with EMR. A decision to repeat RFA treatment was based on histology rather than visual clearance of disease. At 12 mo after the first ablation, biopsies were again taken to assess for eradication of dysplasia (CR-D) and this was defined as the primary end point. Patients with residual disease at this point were considered for ongoing endotherapy at the clinicians' discretion after consultation with the patient and discussion at the sMDT. Development of invasive cancer at any time was defined as treatment failure and data were censored at this point. An overview of the study protocol is shown in Figure 2.

### Radiofrequency ablation procedures and follow up procedures

RFA was delivered circumferentially (HALO 360) or focally (HALO 90) at 12 J/cm<sup>2</sup>. As opposed to the 2 ablations delivered for RFA in BE at each treatment session, only a single ablation was delivered in the registry protocol for squamous HGD. In patients with multifocal dysplasia circumferential RFA was applied and focal RFA administered in patients with unifocal well defined areas of dysplasia. These areas were reported in centimeters from the incisors so that follow up procedures could use this reference to interrogate treated segments of esophagus for success or failure.

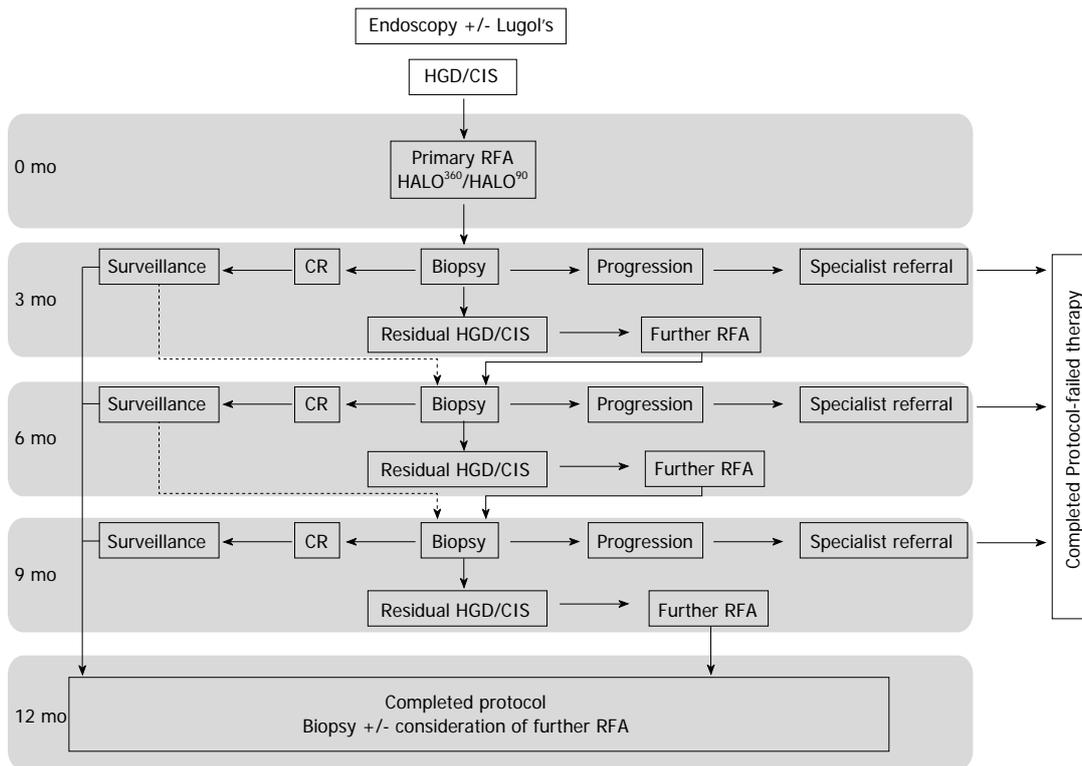


Figure 2 HALO radiofrequency ablation protocol for ablation of Squamous high grade dysplasia and carcinoma *in situ*. Endoscopic mucosal resection (EMR) was carried out at any stage for new visible lesions. RFA: Radiofrequency ablation; HGD: High grade dysplasia; CIS: Carcinoma in situ; CR: Complete reversal.

Chromoendoscopy with magnifying endoscopy and enhanced imaging were used at follow up procedures to examine the previously treated areas referenced at the previous endoscopy. Again all histology was reviewed by two expert gastrointestinal histopathologists.

**Post procedure care/follow up**

All patients were maintained on a twice-daily regimen of a proton pump inhibitor. Soluble co-codamol was prescribed for discomfort post procedure. All patients were discharged home the same day after review by the endoscopist. Follow up endoscopies were carried out at 3 monthly intervals as per protocol with enhanced endoscopic imaging and lugol’s chromoendoscopy (Figure 2).

**Primary and secondary end points and long term outcomes**

The primary end points were complete reversal of squamous dysplasia (CR-D) at 12 mo and development of invasive cancer at any stage. Secondary end points include long-term durability, number of RFA procedures and adverse events. We also followed up all patients who progressed to cancer so that we could determine their long-term outcomes.

**Statistical analysis**

Endpoints such as CR-D at end of protocol were compared to the patient’s baseline status using log rank test and long term outcomes predicted with Kaplan-Meier survival analysis.

**RESULTS**

Twenty seven patients were treated at 8 different centers nationwide. Twenty of these have completed the treatment protocol and we report the outcomes of these patients.

Pre treatment parameters are given in Table 1. Four patients (20%) had disease limited to 1cm (focal disease) and the remaining 16 patients had definable lengths of dysplasia (multi-focal disease) with a median length of 5cm (IQR 1-10). Five patients (25%) had EMR before starting RFA treatment. All patients gave informed written consent. Median follow up for those who had successful ablation and are still in follow up (*n* = 10) is 24 mo (IQR 17-54).

Patients had a median of 1 RFA session and mean of 1.5 RFA treatments during the protocol period. A total of 6 rescue EMRs have been carried out in 6 patients after their first RFA. Two of these patients had already undergone EMR prior to initiating RFA treatment.

**Reversal of dysplasia at end of protocol**

Ten patients (50%) had reversal of dysplasia/CIS (CR-D) at end of protocol after a median of 1 treatment. Eight of these patients (80%) remain free of dysplasia on their latest follow up (median follow up from first treatment 24 mo, range 19-54 mo, see Figure 3). Of the 2 patients who had a recurrence after initial successful RFA, one progressed to invasive disease. The other had multifocal low grade dysplasia (LGD) 4 mo after completing treatment and at latest follow up (41 mo after protocol end) has had 3 further circumferential ablations and one focal

**Table 1** Demographic data of all patients in squamous high grade dysplasia, carcinoma in situ United Kingdom radiofrequency ablation registry *n* (%)

Variable	Value
No. of patients completed protocol	20
Age, yr	
mean $\pm$ SD	71.6 $\pm$ 9.4
Range	38-88
Sex,	
Female	16 (80)
Male	4 (20)
Caucasian ethnicity,	20 (100)
Grade of esophageal neoplasia at study entry	
High grade dysplasia (Tis)	12 (60)
ESCC (T1a)	8 (40)
1Focal squamous dysplasia	4 (20)
2Multi-focal squamous dysplasia	16 (80)
Length of non-focal squamous dysplasia, cm	
mean $\pm$ SD	6.1 $\pm$ 4.2
Number of centers recruiting	8
Previous endoscopic mucosal resection	5 (25)

<sup>1</sup>Focal dysplasia refers to single plaques of dysplasia 1 cm or less in length;

<sup>2</sup>Multi-focal dysplasia refers to multiple plaques of dysplasia > 1 cm in length. ESCC: Early squamous cell carcinoma.

ablation. This patient still has LGD at latest follow up. The clinician and patient have, nonetheless, agreed to perform a further RFA treatment.

Four patients (20%) had residual dysplasia at 12 mo. One patient was referred for surgery to remove the dysplasia. One patient left the country and has been lost to follow up. The other 2 have opted to have further RFA and after a mean of 2 RFA treatments over 10 mo are free of disease at present (Figure 3). Figure 4 shows the predicted rate of dysplasia reversal in all 20 patients who have undergone treatment and completed protocol using Kaplan Meier outcome statistics.

#### Previous endoscopic resection and baseline histology

Although our numbers are too small to draw firm conclusions, there is a trend towards baseline histological grade (HGD or ESCC) having an influence on eventual outcome with HGD having a better outcome (66% *vs* 33%, HR = 0.535, *P* = 0.0308, 95%CI: 0.141-1.505, Log rank test). Larger numbers are however needed to confirm this trend. Our data do not suggest that EMR before commencing RFA influences dysplasia reversal rates and long term outcomes (33% *vs* 66%, HR = 0.229, *P* = 0.778, 95%CI: 0.3264-3.642, *P* = 0.8882, Log Rank test). Dysplasia reversal was also the same whether EMR was required during the RFA protocol (3/6 - 50%) or whether patients underwent RFA alone (7/14 - 50%).

#### Early cancer progression and overall cumulative risk of progression

Four of the 20 (20%) patients had progressed to invasive SCC at their first follow up and therefore no further RFA was performed. Two further patients who were treated with an initial circumferential RFA followed by an EMR at follow up endoscopy progressed to invasive cancer

at protocol end. All progressors were referred for consideration of chemoradiotherapy. Using Kaplan Meier analysis, the risk of progression to invasive disease in all 20 patients who completed the protocol is 26% at 18 mo (Figure 5).

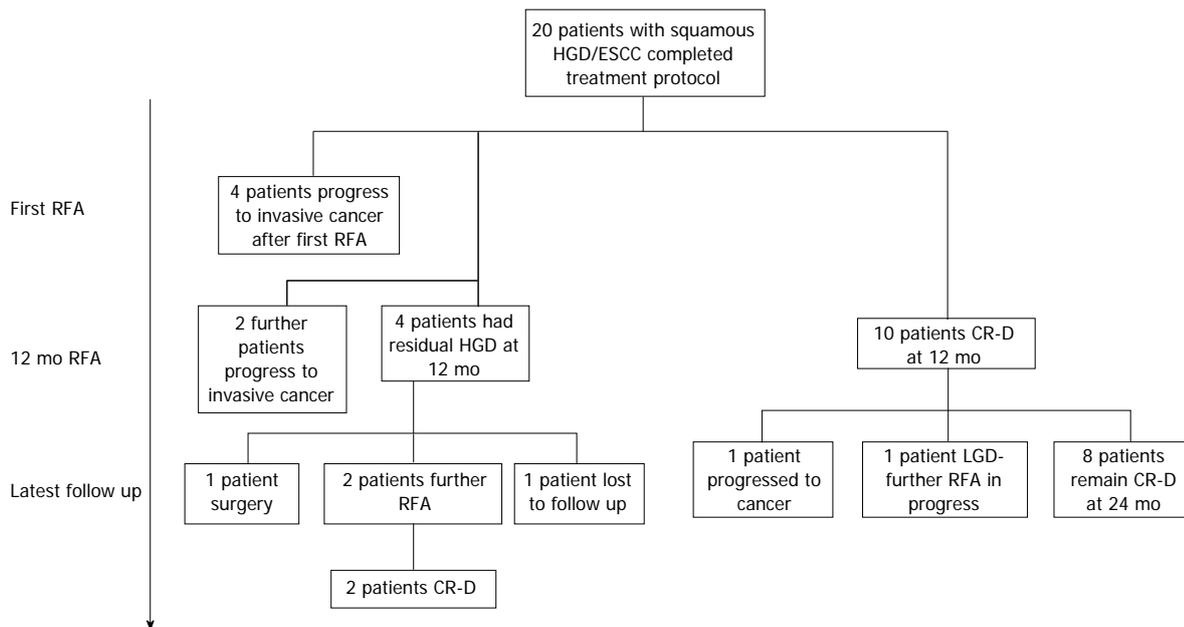
#### Adverse events

One patient suffered a superficial esophageal tear following sizing prior to attempted circumferential ablation. The procedure was discontinued and focal ablation was used at the same procedure. The patient was discharged home without any complications. Four patients (20%) required dilatations for moderate esophageal structuring after their first circumferential treatment. Only one had been treated by EMR prior to RFA. Two of these patients have required serial dilatations thereafter for symptomatic dysphagia with a median of 4 dilatations per patient. The other 2 patients required only a single dilatation to achieve an adequate symptomatic response. Three serious adverse events have been reported to date. Two patients had bleeding at their follow up endoscopy after biopsies and required adrenaline injection. Both occurred following Lugol's iodine application. Although they were admitted overnight in hospital for observation they were discharged the following day without blood transfusion.

## DISCUSSION

The role for endotherapy such as RFA as a first line intervention for patients with squamous HGD and ESCC *in situ* is yet to be established as standard practice. Squamous dysplasia is a very aggressive pathology and early diagnosis and intervention are paramount as disease progression often precludes curative therapy. However the high surgical mortality rate of up to 2%-5% and subsequent morbidity of 20%-50% means that alternative minimally invasive and novel techniques must be investigated<sup>[5,11,12,31]</sup>.

Early literature into the use of RFA in squamous dysplasia emerged in 2008, following on from its recognized potential in early Barrett's neoplasia. Pouw *et al*<sup>[32]</sup> described the case of a 66-year-old patient with a unifocal lesion within the esophagus. This lesion had arisen after the patient had previously undergone chemoradiotherapy for a T2N1M0 squamous cell cancer of the hypopharynx. Following pre-treatment staging to ensure the lesion was confined to the mucosal surface only, the patient received a single balloon based ablation and had no recurrent disease at 4 mo follow up. Subsequent to this report, data regarding the efficacy of RFA in squamous dysplasia have been limited. One of the largest series to date examined the success of RFA in 13 patients within two tertiary centers<sup>[28]</sup>. Nine of the study cohort (69%) required EMR at baseline for visible nodules prior to commencing RFA. Dysplasia reversal was excellent in these patients with 100% achieving CR-D with a median of 2 treatments and remaining disease free with a follow up period of 17 mo from first treatment. In this study patients received 2 ablations at each treatment endoscopy with 12 J/cm<sup>2</sup> for the circumferential ablation and 15 J/cm<sup>2</sup> for focal



**Figure 3** Outcomes for all 20 patients who have completed treatment protocol with radiofrequency ablation for squamous high grade dysplasia/early squamous cell carcinoma. RFA: Radiofrequency ablation; HGD: High grade dysplasia; ESCC: Early squamous cell carcinoma; CR-D: Complete reversal dysplasia; LGD: Low grade dysplasia.

ablation. Stenosis and stricturing was confined to just 2 of the 13 patients in this series. This same group has recently gone on to investigate the efficacy of RFA in a larger cohort of 29 patients in a prospective study<sup>[33]</sup>. This study was conducted in a single Chinese center. All patients underwent an index circumferential ablation of all unstained lesions (USLs) with Lugol’s chromoendoscopy. All patients were followed up at 3 monthly intervals with chromoendoscopy with biopsies followed by focal ablation of any USLs. Using the Chinese classification of squamous neoplasia<sup>[34]</sup>, 18 patients had moderate intraepithelial neoplasia (MGIN), 10 had high grade intraepithelial neoplasia (HGIN), and a single patient had early ESCC. At 12 mo 97% (28/29) had a complete reversal of neoplasia and furthermore there was no progression to invasive cancer within the treated group. The single patient with residual disease at 12 mo had EMR for unifocal disease with clear resection margins.

In our study examining outcomes from the UK RFA registry of patients with squamous dysplasia undergoing RFA, CR-D was 50% at protocol completion, although dysplasia was later reversed in two further patients following more RFA sessions. These figures are lower than the limited published data from other centers worldwide<sup>[28]</sup>. We designed our study with a protocol end at 12 mo so that this study could be directly compared with those previously published. It may be that future studies should consider alternative end points to allow for a longer duration of treatment.

In our series 20% of patients progressed to invasive disease after only one session of RFA and were then offered chemo-radiotherapy. These patients represent most of those who eventually developed cancer. This suggests that a single RFA treatment might even be considered as

a staging procedure. Early failure would identify patients who should be treated with more conventional modalities. Indeed, 80% of those who achieved successful reversal of dysplasia at the end of the RFA treatment protocol remain in remission at most recent follow up.

The 20% rate of progression after a single treatment may also point to the fact that these patients may have been under staged and may in some cases harbor more aggressive neoplasia at baseline that was not sampled. Whereas with other dysplastic conditions of the esophagus such as BE where there are often distinct areas of abnormality within the macroscopically visible columnar lined mucosa, with squamous dysplasia these areas are subtle. Detection relies on adjuncts such as chromoendoscopy with Lugol’s iodine solution, optical endoscopic enhancements and the experience and expertise of the endoscopists to spot anomalies. Even with Lugol’s solution the accuracy of detecting lesions varies greatly. In a recently published series the positive predictive value for Lugol’s detecting squamous neoplasia in unstained lesions after RFA was only 14%<sup>[33]</sup>.

It appears that the use of EMR in our cohort of patients is somewhat limited compared to similar published studies<sup>[15,35,36]</sup>. Only 5 patients (25%) underwent EMR before starting HALO RFA and there were a total of only 6 resections in 4 patients after their index treatment. This may account for the lower rates of dysplasia clearance and progression after the index treatment. Visible nodular lesions before or after RFA treatment may harbor submucosal disease and unless resected early may represent recurrence and progressive invasive disease.

The published data on the success of RFA in BE is robust and plentiful whilst there is limited data on its use in squamous HGD. The AIM dysplasia trial<sup>[26]</sup> demon-

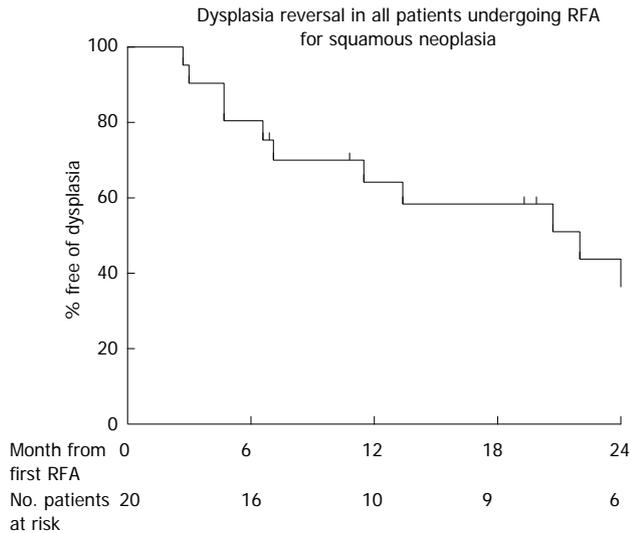


Figure 4 Kaplan Meier outcome statistics predicting the risk of recurrent dysplasia and durability of radiofrequency ablation treatment in all 20 patients treated with radiofrequency ablation. RFA: Radiofrequency ablation.

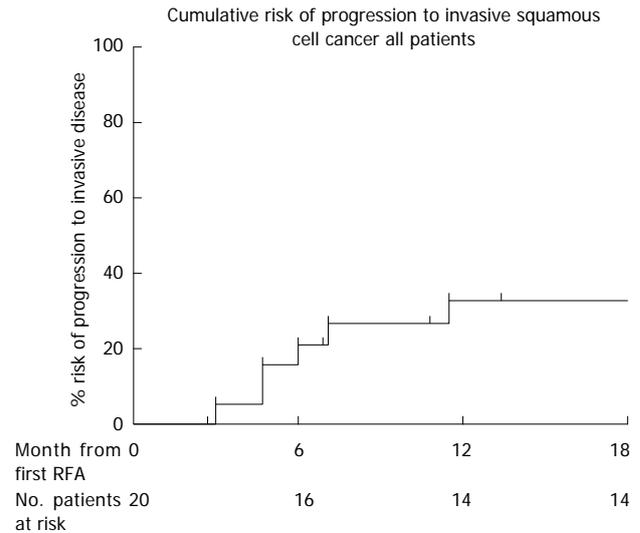


Figure 5 Cumulative risk of cancer progression in all 20 patients who completed treatment with radiofrequency ablation for high grade dysplasia/early squamous cell carcinoma in the United Kingdom radiofrequency ablation registry. RFA: Radiofrequency ablation.

strated impressive outcomes with reversal of HGD in patients with BE as high as 81% with a structured ablation protocol over 12 mo. In this protocol all patients were ablated twice at each treatment with 12 J/cm<sup>2</sup> for both circumferential and focal ablation. Mean treatments per patient was 3.5 ablations and stricturing occurred in 5% requiring dilatations. Subsequent published literature and our own outcomes from the RFA United Kingdom national registry have reproduced similar outcomes in patients with BE<sup>[37]</sup>. In 335 patients with Barrett’s related neoplasia, HGD was cleared from 86% of patients, all dysplasia from 81%, and BE from 62%, at the 12-mo time point, following a mean 2.5 RFA procedures.

There is still debate about the number of ablations patients with squamous dysplasia should undergo. It is also not clear whether these should be performed at successive treatment encounters or whether these patients should undergo staging with chromoendoscopy after successive treatments. In our series, the median number of ablations was only one (range 1-3) for the 10 patients who achieved CR-D at 12 mo. These were all circumferential balloon ablations. This compares to a median of 3 ablations administered in patients with BE during a similar treatment protocol in published series. This may account for the lower eradication rates but the protocol was designed to provide an extremely cautious approach to restaging these patients after every treatment in view of the aggressive nature of the disease.

The rate of stricturing in our series was 20%. All 4 patients who developed strictures had undergone circumferential balloon ablation with the HALO 360 device. This rate is higher than noted in trials of ablation for Barrett’s oesophagus<sup>[27,28]</sup>. Nonetheless they were all overcome with straightforward dilatations. In a recent series of patients treated with RFA for squamous dysplasia the rate of stricturing was 14% after circumferential ablation<sup>[33]</sup>. Other than a self-limiting bleed from biopsies

and not from a RFA procedure our study confirms that RFA is a safe procedure with few other reported adverse events.

A criticism of the protocol in the United Kingdom registry is that there may be too long an interval between treatments due to the requirement of a mapping endoscopy every 3 mo after RFA. With the aggressive nature of this disease it is very important to restage these patients early after treatment and perhaps shortening the intervals between treatment and follow up may help improve outcomes. Current United Kingdom practice is to only ablate once at each treatment session for patients with squamous neoplasia compared to the double ablation carried out in BE with mucosal cleaning of the coagulum between treatments. Recent publications have used 2 ablations per session safely in patients with squamous dysplasia and our practice may have to change to improve outcomes. Delivering 2 ablations at a single treatment session is standard practice for patients undergoing RFA for BE. Bergman and colleagues<sup>[33]</sup> explored various ablation energy settings in patients with squamous dysplasia. These included 2 ablations at a single treatment session with and without coagulum clearance between ablations. In 16 cases where they employed a 12 J/cm<sup>2</sup> - clean - 12 J/cm<sup>2</sup> protocol CR-D at 12 mo was 100% with a stricture rate of 19% compared to a CR-D of 86% and stricture rate of 14% with a 12 J/cm<sup>2</sup> - no clean - 12 J/cm<sup>2</sup> ablation protocol. By using lower energy settings of 10 J/cm<sup>2</sup> for the second ablation or using 10 J/cm<sup>2</sup> for delivering both ablations they did not compromise CR-D (100%) but interestingly had no strictures. The numbers in each of these treatment groups were however low (4 and 2 cases respectively).

Another short coming of our study is the small size of the cohort. These lesions are rarely diagnosed at an early stage. With the availability of high definition en-

doscopy and growing experience of minimally invasive endotherapy as an alternative to surgical and oncological interventions, these numbers will undoubtedly grow in the coming years.

This study examines data from 8 different centers nationwide. Despite standardized protocols, the expertise and experience of each endoscopist will be very different and individual preference and clinical practice will differ from one center to another. These patients have all undergone endotherapy within the confines of demanding endoscopy service provision. These data represent real life outcomes of integrating novel and ground breaking endotherapy to existing practice.

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## COMMENTS

### Background

Oesophageal cancer carries a poor prognosis as it is often diagnosed at a stage where curative therapy is no longer possible. Squamous cell cancer (SCC) of the oesophagus carries a 5-year survival of 10%-15%. The precursor lesion to SCC is squamous dysplasia. Treatment of these early lesions with endoluminal therapy may help to improve outcomes in these high risk patients.

### Research frontiers

Radiofrequency ablation (RFA) is a novel and minimally invasive field ablation technique that has shown good safety and efficacy for treating patients with Barrett's related neoplasia in the oesophagus. By combining endoscopic mucosal resection for visible lesions and RFA, patients with early squamous neoplasia of the oesophagus may be treated at an early stage of the disease.

### Innovations and breakthroughs

There are only limited series reporting the use of RFA in patients with early squamous neoplasia to date. These data better inform us on patients that are likely to succeed with endoscopic therapy but also the importance of careful staging in these patients before treatment.

### Applications

This prospective study may better inform clinicians about minimally invasive endoscopic therapy in these patients where perhaps more radical treatments are not an option for patients.

### Peer review

The authors present a good study to evaluate the role of radiofrequency ablation in early squamous cell cancer and high grade squamous dysplasia. They provide a new finding of early response to RFA is prognostically important. This could be clinically relevant for physicians to perform RFA in the management of squamous high-grade dysplasia.

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## Disparities of conjugating protective enzyme activities in the colon of patients with adenomas and carcinomas

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### Abstract

**AIM:** To investigate the metabolic enzymatic capacity of the colon mucosa to detoxify noxious carcinogenic compounds.

**METHODS:** We investigated the activity of 2 conjugating enzymes-the microsomal uridine glucuronosyltransferase (UGT) and the cytosomal glutathione S-transferase (GST) in the uninjured mucosa of the colon transversum and sigmoideum in patients with adenomatous polyps and colorectal cancer. Biopsies were taken from the mucosa during colonoscopies which were done for clinical (diagnostic) reasons. After storage, the biopsy material was homogenized and after differential centrifugation the enzyme assays were performed with 4-nitrophenol (UGT) and 1-chloro 2,4-dinitrobenzene (GST) as substrates.

**RESULTS:** About 48 patients were included of which

28 had adenomas and 20 had colorectal carcinomas confirmed by histopathology. Enzyme activities were expressed as nmol/mg per minute protein for the GST and as pmol/mg per minute protein for the UGT. Analysis of variance (*F*-test) indicated that both enzymes were more widely distributed in adenoma than in cancer patients. The means  $\pm$  SD were smaller for cancer patients: GST for adenomas  $268 \pm 152$  vs  $241 \pm 69$  for carcinomas and UGT for adenomas  $197 \pm 200$  vs  $150 \pm 86$  for carcinomas.

**CONCLUSION:** Compared to patients with adenomatous colon polyps those with colorectal carcinoma exhibited a lower capacity of detoxifying enzyme metabolism and their activities clustered over a smaller range.

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**Key words:** Glutathione S-transferase; UDP-glucuronosyltransferase; Detoxification; Colon adenoma; Colon carcinoma

**Core tip:** Protective enzymes can conjugate carcinogenic chemicals. The functional capacity of these enzymes is diminished in patients with colorectal cancer and in some patients with colon adenomas.

Hoensch HP, Roelofs HMJ, Edler L, Kirch W, Peters WHM. Disparities of conjugating protective enzyme activities in the colon of patients with adenomas and carcinomas. *World J Gastroenterol* 2013; 19(36): 6020-6025 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6020.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6020>

### INTRODUCTION

Colorectal cancer is an important cause of cancer death in Western countries. In Europe, colorectal cancer (CRC) is the second cause of death from malignant disease

after lung cancer<sup>[1]</sup>. It is estimated that up to 10% of CRC cases can be attributed to hereditary factors leaving approximately 90% of so called sporadic colorectal cancer cases, which may be attributed to diet and lifestyle factors<sup>[2]</sup>. Epidemiological studies have shown the importance of dietary habits in the risk for CRC. Diets low in fruit and vegetables, and high in red meat and fat are associated with an increased risk of CRC<sup>[3,4]</sup>. Humans may be daily exposed to a large variety of toxic or even carcinogenic compounds, present in food<sup>[5]</sup> or as a result of lifestyle habits such as smoking or use of alcohol<sup>[6,7]</sup>. However, humans possess a highly efficient system of defense against such harmful compounds. Detoxification enzymes such as UDP-glucuronosyltransferases (UGTs)<sup>[8]</sup> and glutathione S-transferases (GSTs)<sup>[9]</sup> are responsible for the efficient modification and detoxification of harmful molecules. These enzymes are present in many tissues and also in the gastrointestinal tract in esophagus, stomach, small intestine, large intestine and in the liver<sup>[8-11]</sup>. UDP-glucuronosyltransferases catalyze the conjugation with glucuronic acid of a wide variety of exogenous compounds (*e.g.*, drugs, pesticides, tobacco smoke components such as benzo(a)pyrene) as well as endogenous compounds (*e.g.*, bilirubin, bile acids, steroid hormones)<sup>[8]</sup>. Glutathione S-transferases catalyze the reaction of glutathione with mainly exogenous electrophiles (*e.g.*, polycyclic aromatic hydrocarbons, heterocyclic amines) and endogenous products of oxidative stress<sup>[9]</sup>. The conjugates formed by these enzyme reactions are generally less toxic than their precursors and are more water-soluble, which facilitates their biliary and renal excretion. The gastrointestinal tract is in direct contact with potentially toxic or (pre) carcinogenic agents, ingested by food, medication, drugs, *etc.* and the intestinal mucosa acts as a first-line barrier<sup>[12]</sup>. Tissue-specific expression of the different isoforms of GSTs and UGTs in colon and liver was demonstrated to result in the differences in enzyme activities as measured in these tissues<sup>[10,11,13,14]</sup>.

Earlier, we demonstrated an inverse relationship between GST enzyme activity and cancer risk in several organs of the gastrointestinal tract<sup>[15]</sup>, suggesting that the levels of phase II detoxification enzymes could be pivotal in cancer prevention. After comparison of detoxification levels in small intestine (a site of low cancer risk) and large intestine (high cancer risk), we even postulated that the levels of detoxification enzymes in the colon could be critically low<sup>[16]</sup>.

We now investigated mucosal GST and UGT detoxification activities in normal mucosa of patients with colorectal adenomas, which are at risk to develop colorectal cancer, and in patients who already did develop colorectal cancer. Individual susceptibility to CRC could be partly due to low levels of detoxification enzymes.

## MATERIALS AND METHODS

### Patients

Fifty-one patients gave their written informed consent to use additional biopsy material for this study, the protocol

of which was approved by the Ethics Committee of the university of Dresden/Germany. Colonoscopies were performed exclusively for clinical (diagnostic) reasons in all patients and colorectal cancer or adenomas were confirmed in  $n = 48$  patients. Figure 1 shows the characteristics of the 48 patients included in the statistical analyses of the study. Endoscopic findings were confirmed by histopathology using a standard protocol. Information on the clinical variables was taken from the patients' clinical files. Consecutive patients with adenomatous polyps or colorectal cancers were included regardless of localization, size, stage and histological grading. All patients with neoplasia of the colon were included if there was pathologically proven neoplasia. We excluded patients with insufficient clinical data and those without biopsies of the uninvolved mucosa.

### Methods

Forceps biopsies of the colon mucosa were taken from the uninvolved mucosa of the transversum and the sigmoid colon. Biopsy material was shock-frozen and stored in liquid nitrogen until analysis. Biopsies were weighed and homogenized by 15 strokes in a plastic/plastic potter in 5 volumes of a buffer solution (pH = 7.4) containing 0.25 mol/L saccharose, 20 mmol/L Tris/HCl and 1 mmol/L dithiothreitol (all chemicals were from Sigma, Zwijndrecht, The Netherlands). Half of the total homogenate was frozen at  $-80^{\circ}\text{C}$  in small aliquots and used for the UGT assay. The other half of the homogenate was used for preparation of cytosol by centrifugation for 60 min at 150000  $g$  and at  $4^{\circ}\text{C}$  in a 42.2Ti rotor (Beckman Optima L-70K). The supernatant (cytosol) was frozen at  $-80^{\circ}\text{C}$  in small aliquots and used for the GST assay. Protein content was determined in cytosol ( $2 \times 5 \mu\text{L}$ ) and homogenate ( $2 \times 5 \mu\text{L}$ ) and GST enzyme activity in the cytosolic fractions ( $2 \times 10 \mu\text{L}$ ) was determined with 1-chloro-2,4-dinitrobenzene as substrate as described before<sup>[17]</sup>. UGT enzyme activity in the homogenates ( $1 \times 20 \mu\text{L}$ ) was measured with 4-nitrophenol as substrate according to Strassburg *et al.*<sup>[18]</sup> Usually, 2-3 biopsy particles per patient were taken from each site (uninvolved colon transversum and colon sigmoideum). For some patients not enough biopsy material was obtained to perform the complete set of enzyme assays (Figure 1). Combined activity was calculated as the mean of the activity of sigmoid and transversum when both locations were biopsied per patient, otherwise as the activity of one of the two locations only.

### Statistical analysis

Patient characteristics were analyzed using descriptive statistical methods. Enzyme activities in the different groups analyzed were described using means and standard deviations and compared using the  $t$ -test. An  $F$ -test was used to compare the variances in the two groups. The correlation between GST and UGT was described numerically using the Spearman correlation coefficient and graphically using linear regression exhibiting both confidence intervals (for the means) and prediction intervals for the

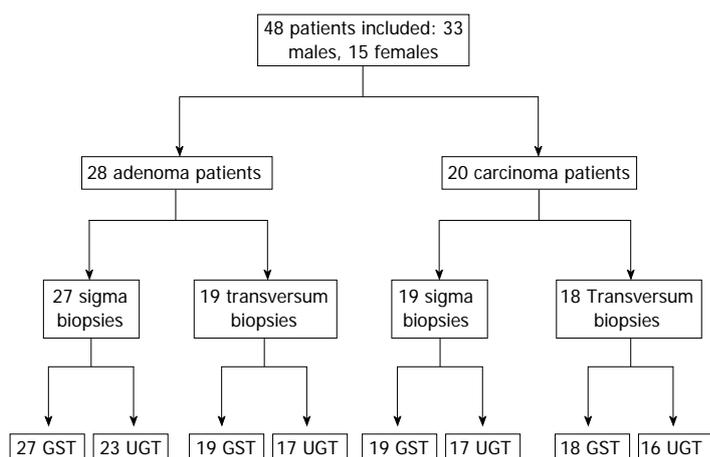


Figure 1 Flow-chart: Glutathione S-transferase- and uridine glucuronosyltransferase activities examined from biopsies of adenoma and carcinoma patients. The number of patients in each type of neoplasia, the number of biopsy sites and the number and type of enzyme assays performed per site are shown. GST: Glutathione S-transferase; UGT: Uridine glucuronosyltransferase.

Table 1 Patient characteristics *n* (%)

Characteristics	Colorectal cancer ( <i>n</i> = 20)	Adenomas ( <i>n</i> = 28)	<i>P</i> -value
Sex			0.001 <sup>1</sup>
Male	9 (45)	24 (86)	
Female	11 (55)	4 (14)	
Age, yr, mean (min-max)	68.4 (39-86)	68.4 (51-88)	NS <sup>2</sup>
Alcohol			0.021 <sup>1</sup>
Yes	3 (16)	15 (54)	
No	17 (84)	13 (46)	
Smoking			NS <sup>1</sup>
Yes	3 (16)	2 (16)	
No	17 (84)	26 (84)	
Aspirin			NS <sup>1</sup>
Yes	2 (10)	5 (18)	
No	18 (90)	23 (82)	
NSAIDs			NS <sup>1</sup>
Yes	20 (100)	27 (97)	
No			
BMI (kg/m <sup>2</sup> )	25.1	25.6	NS <sup>2</sup>

Distribution of clinical factors among patients with carcinomas and adenomas of the colon. <sup>1</sup> $\chi^2$  test; <sup>2</sup>Wilcoxon Rank Sum test. NSAID: Nonsteroidal anti-inflammatory drugs; BMI: Body mass index; NS: Not significant.

actual values. SAS/STAT software, Version 9.3 was used for all statistical analyses. *P*-values  $\geq 0.05$  were identified as not significant (NS) throughout this paper.

## RESULTS

We investigated whether patients with adenomas or CRC differed in their colon capacity to metabolize noxious chemical compounds using the activities of detoxifying conjugating phase II enzymes as biomarkers. Table 1 shows the patients' demographic characteristics: 20 patients had colorectal carcinomas with stage 2 and 3 and 28 patients had adenomatous polyps of various location, size and histological types. In 3 patients the

Table 2 Enzyme activities of glutathione S-transferase and uridine glucuronosyltransferase in the normal colon mucosa of patients with adenoma and carcinoma (mean  $\pm$  SD)

Enzyme activities	Adenomas ( <i>n</i> = 28)	Carcinomas ( <i>n</i> = 20)	<i>P</i> -value ( <i>F</i> -test)
GST (nmol/mg per minute)			
Transversum	225 $\pm$ 104 (19)	237 $\pm$ 72 (18)	0.1342
Sigmoid	265 $\pm$ 152 (27)	232 $\pm$ 78 (19)	0.0046
Combined	268 $\pm$ 152 (28)	241 $\pm$ 69 (20)	0.0007
UGT (pmol mg per minute)			
Transversum	231 $\pm$ 269 (17)	159 $\pm$ 122 (16)	0.0015
Sigmoid	170 $\pm$ 158 (23)	144 $\pm$ 96 (17)	0.0456
Combined	197 $\pm$ 200 (27)	150 $\pm$ 86 (19)	0.0005

Number of enzyme assays given in brackets. GST: Glutathione S-transferase; UGT: Uridine glucuronosyltransferase.

diagnosis remained unclear as to the type of neoplasia. The means for GST enzyme activity were lower in the cancer patients than in adenoma patients (except for the transversum), but this difference did not reach statistical significance (Table 2). The UGT means were also lower in cancer patients even when distinguishing between the transversal and sigmoidal location. While the means of enzyme activities were not significantly different when using a t-test it was obvious that the ranges and the standard deviations were much wider for the adenoma patients. Adenoma patients had a wider distribution and cancer patients aggregated at a lower level and over a smaller range. This difference, indicated by the size of the standard deviations could be manifested by using the *F*-test which showed a statistically significant difference for UGT both in the transversum and sigmoid and for GST in the sigmoid. Figure 2 shows the individual distribution of the UGT and GST enzyme activity in the colon transversum of adenoma patients (left panel) and CRC patients (right panel) in the form of a regression analysis with the 95% CIs (for the means) and prediction intervals (for the actual values). The range was narrower for the cancer patients. No statistically significant differ-

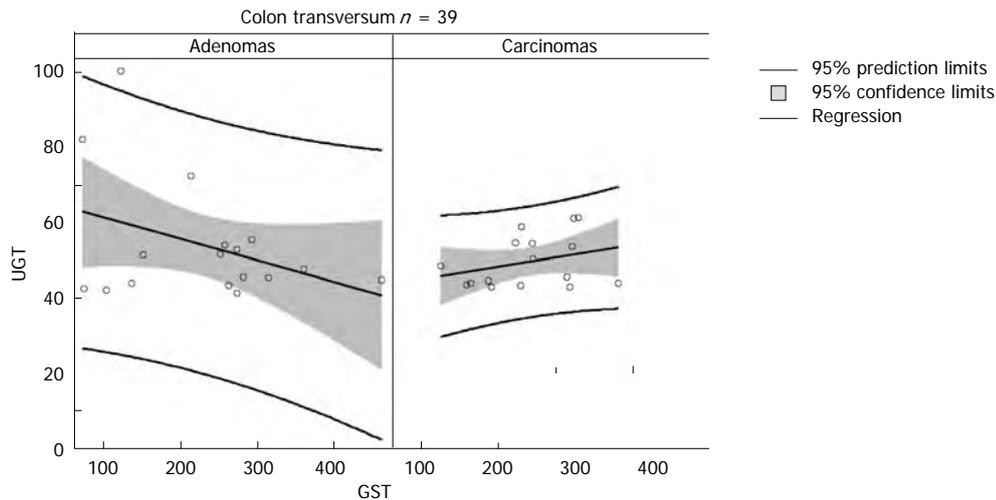


Figure 2 Distribution of enzyme values of uridine glucuronosyltransferase and glutathione S-transferase in the colon mucosa of the transversum of adenoma and carcinoma patients. The correlation between uridine glucuronosyltransferase (UGT) and glutathione S-transferase (GST) over the range of enzyme activities is illustrated in the colon transversum of 39 patients.

**Table 3 Gender differences between glutathione s-transferase and uridine glucuronosyltransferase activities in the colon of patients with colon-neoplasia**

	Male	Female	t-test	F-test
GST (combined)	258 ± 139 (36)	235 ± 68 (15)	0.54	0.006
Transversum	218 ± 94 (26)	251 ± 71 (13)	0.28	0.310
Sigmoid	267 ± 137 (34)	202 ± 75 (14)	0.10	0.020
UGT (combined)	198 ± 180 (34)	146 ± 99 (15)	0.30	0.020
Transversum	231 ± 238 (23)	153 ± 108 (12)	0.29	0.009
Sigmoid	168 ± 142 (29)	141 ± 110 (13)	0.54	0.360

Number of enzyme assays in brackets. For the *t*-test and the *F*-test *P*-values are indicated. GST: Glutathione S-transferase; UGT: Uridine glucuronosyltransferase.

ences of enzyme levels were found between the transversum and sigma, both, for UGT and GST. For this reason the means from the transversum and the sigma were also pooled for the analysis of the combined enzyme levels.

The enzyme activities could be influenced by a variety of clinical factors. Using the clinical charts we examined the major clinical variables to find out if these were affecting the enzyme levels. Both enzymes activities were not influenced by age, alcohol and nicotin consumption, use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) and body mass index (BMI). Female patients had lower enzyme levels and less variability than males (Table 3). There were more males than females among the polyp patients (24 *vs* 4) than among the cancer patients (9 *vs* 11). Also, alcohol consumption was more prevalent in the polyp patients (15 *vs* 13) than in the cancer patients (3 *vs* 17). No differences were detected between both groups for nicotin use, aspirin or other NSAID medications and body weight.

## DISCUSSION

We measured the kinetic detoxifying enzyme activity in

the uninvolved normal appearing mucosa of patients with benign and malign neoplasia of the colon. Usually enzyme values can be expressed using mRNA and protein levels, but these parameters do not reflect the functional activity within the epithelial cells of the mucosa. Using kinetic data we could obtain direct information on the metabolic capacity of the tissue. Both UGT and GST had a significantly different distribution between patients with benign neoplasia (adenomas) and those with cancerous neoplasia (CRC). The type of neoplasia was associated with the enzyme levels and an increased variance was found in the polyp patients. On the other hand the enzyme levels could inform on the degree of neoplasia and its evolution. Cancer patients had a smaller range at a lower level. This could mean that cancer patients had lost some of the mucosal detoxifying potential, predisposing them to develop cancer. The wide range of enzyme values could indicate that some patients with adenomas who were in the lower range might be at risk to develop cancer. It is possible that adenoma patients who are in the higher range are protected from cancer development since they possess a higher protective enzyme capacity. This hypothesis needs to be validated by longitudinal follow-up studies of enzyme values in polyp patients over long periods of time. Previously, we found in the rectum of healthy controls GST-levels of  $321 \pm 29$  nmol/mg per minute protein ( $n = 10$ )<sup>[19]</sup> which were higher than those reported in this paper.

The UGT enzyme system is located in the endoplasmic reticulum of the mucosal intestinal cells and these enzymes can detoxify hazardous chemical compounds - mostly lipophilic - which penetrate deep into the interior of the cells. The GST enzymes are located within the cytoplasm of the cells and use intracellular glutathione to protect the cells from electrophilic chemicals. Both enzymes perform conjugation reactions and thereby render xenobiotics water-soluble (phase II metabolism). The conjugated metabolites can be readily excreted by

the liver or the kidney. Phase II enzymes can be induced by their substrates and are influenced by environmental chemicals and clinical factors in the human body<sup>[20]</sup>. In contrast to our previous publication<sup>[21]</sup> there were no significant differences of enzyme levels relating to the location between the upper and the lower segments. However, this might be due to the different patient population (mainly normal and inflammatory findings) investigated previously. Female gender was associated with a higher prevalence of carcinomas and lower enzyme activities. This might have contributed to the observed enzyme disparities. One limitation of this study was the small number of patients in the 2 groups. Furthermore due to the small sample size enzyme values at the m-RNA level could not be studied. Further studies should include enzyme measurements at the protein and mRNA level. More patients and addition of a control group with non-neoplastic diseases of the colon should be included.

Our results suggest that protective enzymes could be diminished in the colon mucosa of cancer patients. Reduced activities of protective enzymes could lead to increased susceptibility to develop colorectal cancer.

## ACKNOWLEDGMENTS

We want to thank Sascha Mack for helping us to collect the patient data.

## COMMENTS

### Background

It is still controversial which factors determine the development of colorectal neoplasia. Exogenous dietary agents can be broken down by protective enzymes in the colon mucosa.

### Research frontiers

The cellular mutations of the tumor tissue in colorectal cancer (CRC) are thoroughly investigated but preneoplastic aberrations in the mucosa of the colon that lead to development of neoplasia are rarely investigated. The authors studied the properties of the uninvolved mucosa in patients with colon neoplasia by investigating the activities of protective enzymes.

### Innovations and breakthroughs

The direct comparison of activities of conjugating protective enzymes in carcinoma and adenoma patients has not been reported so far. It is highly informative to find out whether 2 types of neoplasia with different degree of malignant potential exhibit variances in the distribution of enzyme activities. We found that lower enzyme activities were associated with CRC but adenoma patients still retained sufficient activities. Susceptibility to develop CRC could depend on the enzyme levels expressed by the preneoplastic mucosa. Low enzyme levels could be a risk factor for adenoma patients to develop CRC.

### Applications

By inducing protective enzymes with dietary agents it might be feasible to prevent neoplasia in the colon.

### Terminology

Conjugating enzymes represent Phase 2 of drug metabolism. They mainly break down lipophilic carcinogenic compounds.

### Peer review

This paper makes good efforts in trying to understand that, protective enzymes could be diminished in the colon mucosa of cancer patient and reduced activities of these enzymes could lead to increased susceptibility to develop colon cancer.

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## Endoscopic retrograde cholangiopancreatography with rendezvous cannulation reduces pancreatic injury

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### Abstract

**AIM:** To examine whether rendezvous endoscopic retrograde cholangiopancreatography (ERCP) is associated with less pancreatic damage, measured as leakage of proenzymes, than conventional ERCP.

**METHODS:** Patients ( $n = 122$ ) with symptomatic gallstone disease, intact papilla and no ongoing inflammation, were prospectively enrolled in this case-control

designed study. Eighty-one patients were subjected to laparoscopic cholecystectomy and if intraoperative cholangiography suggested common bile duct stones (CBDS), rendezvous ERCP was performed intraoperatively ( $n = 40$ ). Patients with a negative cholangiogram constituted the control group ( $n = 41$ ). Another 41 patients with CBDS, not subjected to surgery, underwent conventional ERCP. Pancreatic proenzymes, procarboxypeptidase B and trypsinogen-2 levels in plasma, were analysed at 0, 4, 8 and 24 h. The proenzymes were determined in-house with a double-antibody enzyme linked immunosorbent assay. Pancreatic amylase was measured by an enzymatic colourimetric modular analyser with the manufacturer's reagents. All samples were blinded at analysis.

**RESULTS:** Post ERCP pancreatitis (PEP) occurred in 3/41 (7%) of the patients cannulated with conventional ERCP and none in the rendezvous group. Increased serum levels indicating pancreatic leakage were significantly higher in the conventional ERCP group compared with the rendezvous ERCP group regarding pancreatic amylase levels in the 4- and 8-h samples ( $P = 0.0015$ ;  $P = 0.03$ ), procarboxypeptidase B in the 4- and 8-h samples ( $P < 0.0001$ ;  $P < 0.0001$ ) and trypsinogen-2 in the 24-hour samples ( $P = 0.03$ ). No differences in these markers were observed in patients treated with rendezvous cannulation technique compared with patients that underwent cholecystectomy alone (control group). Post procedural concentrations of pancreatic amylase and procarboxypeptidase B were significantly correlated with pancreatic duct cannulation and opacification.

**CONCLUSION:** Rendezvous ERCP reduces pancreatic enzyme leakage compared with conventional ERCP cannulation technique. Thus, laparo-endoscopic technique can be recommended with the ambition to minimise the risk for post ERCP pancreatitis.

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**Key words:** Common bile duct stones; Procarboxypeptidase B; Trypsinogen-2; Pancreatic amylase; Intraoperative endoscopic retrograde cholangiopancreatography

**Core tip:** Endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy and stone extraction is a standard procedure in the management of common bile duct stones. Rendezvous ERCP reduces pancreatic enzyme leakage compared with conventional ERCP cannulation technique. Thus laparo-endoscopic technique can be recommended to prevent post ERCP pancreatitis.

Swahn F, Regnér S, Enochsson L, Lundell L, Permert J, Nilsson M, Thorlacius H, Arnelo U. Endoscopic retrograde cholangiopancreatography with rendezvous cannulation reduces pancreatic injury. *World J Gastroenterol* 2013; 19(36): 6026-6034 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6026.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6026>

## INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy and stone extraction is considered a standard procedure in the management of common bile duct stones (CBDS). Currently ERCP is often performed before the cholecystectomy (preoperative ERCP) or after it (postoperative ERCP). An alternative and increasingly appealing strategy is to carry out ERCP during the cholecystectomy (intraoperative ERCP) as a one-step procedure.

Many authors caution against using ERCP to aid surgical intervention in the management of CBDS owing to the risks of procedure-induced pancreatitis and postoperative failure to cannulate the bile duct. Despite more than three decades of efforts to reduce complication rates, post-ERCP pancreatitis (PEP) remains the most frequent, feared and unpredictable complication with an overall incidence ranging from 3% to 15%<sup>[1-5]</sup> whereas failure to achieve deep biliary cannulation varies between 1.5% and 20%<sup>[6,7]</sup>. The most serious of the several known procedure-related risk factors of PEP are closely associated with prolonged and repeated attempts at cannulation and inadvertent contrast filling of the pancreatic duct<sup>[2,8]</sup>.

Rendezvous cannulation involves a combined laparo-endoscopic approach and can be used in conjunction with intraoperative ERCP. The concept was described for the first time in 1993<sup>[9]</sup>. Approaches for and definitions of rendezvous cannulation have varied over the years, but the basic principle remains an antegrade biliary cannulation over the papilla of Vater. If conducted correctly, the rendezvous procedure offers immediate biliary cannulation and avoids inadvertent cannulation of the pancreatic duct<sup>[10]</sup>.

The question underlying our study is whether combining rendezvous cannulation with ERCP might reduce the incidence of PEP. Considering the incidence of PEP,

large study groups are needed to investigate the impact of different ERCP techniques. On the other hand, pancreatic enzyme leakage is a sensitive marker of pancreatic damage related to development of acute pancreatitis and has been used as such in numerous previous studies<sup>[11]</sup>. In the clinical laboratory, pancreatic amylase is the most frequently used early sign of acute pancreatitis<sup>[12-15]</sup>. Procarboxypeptidase B (proCAPB) has been suggested as an even more sensitive marker for acute pancreatitis, while the proenzyme trypsinogen-2 may be a useful indicator for acute pancreatitis as well as revealing the severity of pancreatitis<sup>[16-19]</sup>.

The aim of our prospective case-control study was to investigate whether use of rendezvous cannulation technique could prevent post-ERCP pancreatic damage in patients treated for gallbladder and common bile duct stones. The primary end point was estimated as leakage of pancreatic pro-enzymes (pancreatic amylase, proCAPB and trypsinogen-2) after ERCP.

## MATERIALS AND METHODS

### *Patients and controls*

Between April 2005 and November 2010 patients who fulfilled the inclusion criteria (Table 1) were recruited as they presented at the Department of Surgical Gastroenterology, Karolinska University Hospital. All patients had a physical examination, were screened for baseline serum markers and were examined with abdominal ultrasonography, CT-scan or MRCP to confirm cholecysto- and/or choledocholithiasis and to rule out ongoing pancreatitis and/or cholecystitis. Among these patients we identified three study groups. Regardless of suspicion of CBDS, patients with cholecystolithiasis who were fit for surgery, were prepared for a standard laparoscopic cholecystectomy. According to the results of the intraoperative cholangiography, patients were allocated to one of two groups: rendezvous ERCP group (positive finding of CBDS) or laparoscopic cholecystectomy only (negative finding of CBDS). The third group, which received conventional ERCP, included patients with high suspicion of choledocholithiasis who had already had a cholecystectomy and patients with cholecystolithiasis and CBDS who were unsuitable for surgery. All patients were hospitalised for a minimum of 24 h, had a clinical examination at 4, 8 and 24 h postoperatively, and had a 30-d follow-up interview by telephone. Post ERCP pancreatitis was defined as post-procedural onset of upper abdominal pain lasting more than 24 h and combined with pancreatic amylase concentration in serum of at least three times the upper reference limit<sup>[20]</sup>.

Demographic and procedure-related data were collected prospectively before and after the procedure and follow-up data were noted at 4, 8 and 24 h postoperatively and at 30 d follow-up. All patients gave oral and written informed consent before they entered the study, which was approved by the Regional Research Ethics Committee of Stockholm.

**Table 1** Inclusion and exclusion criteria

Inclusion criteria	
Clinical and radiological findings fulfilling indication for cholecystectomy due to gallstone	
Clinical and radiological findings fulfilling indication for ERCP due to CBDS	
Adult, $\geq 18$ yr	
Informed consent	
Exclusion criteria	
Acute or chronic pancreatitis	
Acute cholangitis	
Acute cholecystitis	
Antiinflammatory medication	
Failure to perform cholangiography	
Pancreatic or biliary cancer	
Prior ERCP with sphincterotomy	
Conversion from laparoscopic to open cholecystectomy	

ERCP: Endoscopic retrograde cholangiopancreatography; CBDS: Common bile duct stones.

### Laparoscopic cholecystectomy

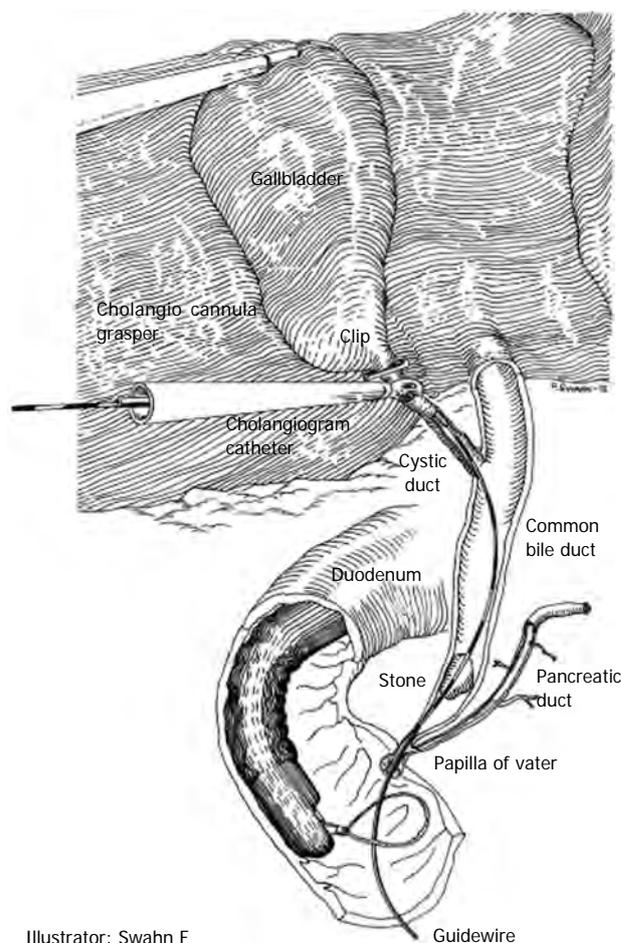
Laparoscopic cholecystectomy was performed according to standard laparoscopic technique with pneumoperitoneum, introduction of four troachars and isolation of the cystic duct. All procedures included intraoperative cholangiography through a small incision of the cystic duct to check for CBDS and to delineate bile duct anatomy.

### Rendezvous ERCP

When cholangiography during laparoscopic cholecystectomy reveals CBDS, the endoscopist is called to the operating room. While waiting for the endoscopist to arrive, the surgeon inserts a transcystic guide wire (Jag-wire™ 450 cm  $\times$  0.0635 cm or 450 cm  $\times$  0.0889 cm, Boston Scientific Corporation, Natick, MA, United States) through the existing cholangiography catheter and advances the guide wire through the choledochus, papilla of Vater into the duodenum. The pneumoperitoneum is deflated to facilitate transoral introduction and positioning of the duodenoscope. For rendezvous cannulation, the guide wire is captured with a polypectomy snare and pulled gently through the working channel of the duodenoscope (Figure 1). When the guide wire is in place, a sphincterotome is introduced over it and the rendezvous cannulation can be completed when the sphincterotome enters the common bile duct. After sphincterotomy, any stones are extracted with retrieval balloons or baskets. The first sweeping is done in choledochus along the transcystic guide wire. Then the guide wire is extracted (with the balloon catheter positioned in choledochus) and repositioned in the intrahepatic ducts to allow clearance of possible remaining supracholedochal stones. If there is any suspicion that stone clearance may be incomplete, a biliary stent is inserted.

### Conventional ERCP

Conventional ERCP was performed with Olympus TJF-160R or TJF-160VR (Olympus Optical, Tokyo, Japan)



Illustrator: Swahn F

**Figure 1** Laparo-endoscopic cannulation technique employing a transcystic guidewire, inserted by the surgeon through a cholangiocannula grasper and captured by the endoscopist with a polypectomy snare in the duodenum.

and wire-guided cannulation (WGC) technique through a sphincterotome (Autotome™ RX 44 Cannulating Sphincterotome, Boston Scientific Corporation, Natick, MA, United States). The complexity of cannulation was rated subjectively by the endoscopist, based particularly on the difficulty of cannulation, the number of inadvertent pancreatic duct cannulations and contrast injections with pancreatic duct filling. If the cannulation required more than six attempts or precut technique, it was considered a difficult cannulation.

All procedures were performed by one of three senior endoscopists with 7-15 years' experience of ERCP and with a present procedure rate of approximately 150-300 ERCP investigations per year. All patients were investigated under general anesthesia. Prophylactic antibiotics were administered as a single intravenous dose of 4 g piperacillin and 0.5 g tazobactam (Tazocin®, Wyeth AB, Solna, Sweden) prior to all procedures. Cholangiography was performed by using Iohexol (Omnipaque®, GE Healthcare, Stockholm, Sweden) monomeric non-ionic contrast medium. Sphincterotomy was carried out with pulsating cutting and coagulation diathermy system (ERBE, Elektromedizin, Tübingen, Germany).

**Table 2** Preoperative demographic characteristics (*n* = 122)

Group	CV ERCP ( <i>n</i> = 41)	RV ERCP ( <i>n</i> = 40)	LC control <sup>2</sup> ( <i>n</i> = 41)	<i>P</i> value
Female	24 (59)	31 (77)	33 (80)	0.06
Age, yr	64 ± 14	47 ± 16	50 ± 15	0.003
Body mass index	29 ± 8.1	27 ± 5.9	27 ± 4.9	0.21
Physical status, ASA-score				0.33
Grade 1	12 (29)	20 (50)	20 (49)	
Grade 2	23 (56)	16 (40)	16 (39)	
Grade 3	6 (15)	4 (10)	5 (12)	
Laboratory values, median (25 <sup>th</sup> -75 <sup>th</sup> )				
Bilirubin, < 26 mmol/L	28.0 (9.0-97.5)	29.0 (10.0-81.0)	9.0 (7.0-16.0)	1.00 <sup>1</sup>
Alkaline phosphatase, < 1.9 µcat/L	3.9 (2.2-5.8)	2.4 (1.6-4.4)	1.8 (1.1-2.8)	0.60 <sup>1</sup>
C-reactive protein, < 10 mg/L	5.0 (5.0-14.0)	5.0 (5.0-5.0)	5.0 (5.0-5.0)	0.54 <sup>1</sup>
Pancreatic amylase, 0.15-1.10 µcat/L	0.4 (0.3-0.7)	0.4 (0.3-0.5)	0.4 (0.3-0.6)	0.56 <sup>1</sup>
Procarboxypeptidase B, nmol/L	7.9 (5.4-16.3)	5.4 (3.5-7.4)	5.3 (3.0-9.5)	0.87 <sup>1</sup>
Trypsinogen-2, 30-110 µg/L	126.0 (83.0-208.4)	118.0 (86.3-132.6)	88.5 (68.6-142.3)	0.39 <sup>1</sup>

Data are expressed as mean ± SD or *n* (%). <sup>1</sup>Conventional endoscopic retrograde cholangiopancreatography (CV ERCP) *vs* rendezvous ERCP (RV ERCP); <sup>2</sup>Laparoscopic cholecystectomy (LC) without ERCP. ASA: American Society of Anesthesiologists.

### Laboratory analysis

Blood samples were collected in ice-cold EDTA (ethylenediaminetetraacetic acid) tubes immediately prior to surgery or endoscopy (0-sample) and sampling repeated at 4 ± 1 h, 8 ± 2 h and 24 ± 4 h after laparoscopic cholecystectomy or ERCP. Plasma was obtained after centrifugation at 2200 r/min for 10 min and then frozen and stored at -70 °C until further analysis.

ProCAPB B and trypsinogen-2 levels in plasma were determined in-house with a double-antibody enzyme linked immunosorbent assay developed at the Department of Surgery, Clinical Sciences, Skåne University Hospital, Malmö<sup>[17,19]</sup>. Pancreatic amylase was measured by an enzymatic colourimetric modular analyser with the manufacturer's reagents (Roche, Diagnostics GmbH, Mannheim, Germany) at the Department of Clinical Chemistry at Karolinska University Hospital in Huddinge, accredited by the Swedish Board for Accreditation and Conformity Assessment. All samples were blinded at analysis.

### Ethics approval

This study was conducted with the approval of the Regional Research Ethics Committee of Stockholm, Sweden.

### Statistical analysis

Power calculations assumed a pancreatic enzyme reaction rate of 14% with the use of the conventional ERCP cannulation technique. Assuming a reduction in pancreatic reaction rate to 1% in the rendezvous group and 1% in the control group, 40 patients in each study arm were required to determine this difference with a 95% probability and with a 75% power ( $\chi^2$  test of equal proportions). The variables pancreatic amylase, proCAPB and trypsinogen-2 were analysed using a mixed linear model with one between-group factor *i.e.*, conventional ERCP, rendezvous ERCP and control group and one within-group factor, time (0, 4, 8 and 24 h) and the subsequent interaction between the factors. In case of significant

interactions, simple main effect tests were performed, *i.e.*, effects of one factor when the level of the other factor was fixed. Results are presented as mean, SD and 95% CIs. The distribution of some variables (*e.g.*, proCAPB, trypsinogen-2, pancreatic amylase) was positively skewed and in these cases the variables were log-transformed before the formal analyses. The binomial response was subsequently analysed by fitting a generalised estimating equations model with the Genmod procedure in SAS<sup>®</sup> System 9.1, SAS Institute Inc., Cary, NC, United States. These latter parameters are presented as OR and 95%CI. *P*-values less than 0.05 were considered statistically significant.

## RESULTS

### Patient characteristics

A total of 139 patients (41 men and 98 women) with a median age of 54 years were enrolled into the study. During the inclusion period 17 patients were excluded due to: acute pancreatitis immediately prior to ERCP (*n* = 3), conversion of the operation from laparoscopic to open cholecystectomy (*n* = 6), tumour (*n* = 4), and retraction of informed consent (*n* = 4), leaving 122 participants for further analyses. Relevant preoperative demographic characteristics (Table 2) did not differ between the three study groups in terms of gender, BMI, physical status according to ASA-classification, baseline laboratory values for CRP, pancreatic amylase, proCAPB and trypsinogen-2. However, there was a difference in age, with the conventional ERCP group being somewhat older. In addition, there was an expected difference between the control group and the other two groups regarding bile duct width, alkaline phosphatase and bilirubin levels but no significant difference between the rendezvous and conventional ERCP group.

### Clinical outcomes

PEP emerged in three (7%) of the 41 patients cannulated with conventional ERCP, *vs* none of those cannulated

**Table 3** Clinical outcome and biometric results at 4-, 8- and 24 h after conventional endoscopic retrograde cholangiopancreatography, rendezvous endoscopic retrograde cholangiopancreatography and laparoscopic cholecystectomy alone (*n* = 122)

Group	CV ERCP ( <i>n</i> = 41)	RV ERCP ( <i>n</i> = 40)	LC control <sup>1</sup> ( <i>n</i> = 41)	<i>P</i> -value
Difficult cannulation, > 6 attempts	9 (22)	0 (0)	NA	0.002 <sup>a</sup>
Precut techniques	6 (15)	0 (0)	NA	0.03 <sup>a</sup>
Cannulation failure	3 (7)	0 (0)	NA	0.24
Pancreatic cannulation	13 (32)	0 (0)	NA	< 0.001 <sup>a</sup>
Pancreatic duct opacification	11 (27)	0 (0)	NA	0.001 <sup>a</sup>
Stone clearance at index procedure	33 (81)	38 (95)	NA	0.01 <sup>a</sup>
Stone size	6.4 ± 5.1	5.5 ± 3.2	NA	0.40
Median endoscopic procedure time, minutes (range)	36 (10-80)	28 (17-55)	NA	0.11
Post ERCP pancreatitis	3 (7)	0 (0)	0 (0)	0.24
Hyperamylasemia, > 3 fold rise above upper reference value, with or without post ERCP pancreatitis	6 (15)	0 (0)	0 (0)	0.03 <sup>a</sup>
Pancreatic amylase (μcat/L), 4 h	2.0 ± 4.2	0.5 ± 0.3	0.4 ± 0.3	0.0015 <sup>a</sup>
Pancreatic amylase (μcat/L), 8 h	3.5 ± 9.0	0.6 ± 0.3	0.5 ± 0.3	0.03 <sup>a</sup>
Pancreatic amylase (μcat/L), 24 h	2.4 ± 6.0	0.6 ± 0.3	0.4 ± 0.2	0.056
Procarboxypeptidase B (nmol/L), 4 h	90.7 ± 237.7	7.2 ± 8.1	6.5 ± 9.3	< 0.0001 <sup>a</sup>
Procarboxypeptidase B (nmol/L), 8 h	111.7 ± 287.3	7.9 ± 7.9	8.0 ± 18.4	< 0.0001 <sup>a</sup>
Procarboxypeptidase B (nmol/L), 24 h	47.4 ± 131.7	8.5 ± 7.9	8.2 ± 9.2	< 0.0001 <sup>a</sup>
Trypsinogen-2 (μg/L) 24 h	317.8 ± 547.1	145.6 ± 67.2	134.0 ± 80.2	0.03 <sup>a</sup>

Data are expressed as mean ± SD or *n* (%). *P*-value indicate differences between conventional endoscopic retrograde cholangiopancreatography (CV ERCP) vs rendezvous ERCP (RV ERCP) and <sup>a</sup>*P* < 0.05. <sup>1</sup>Laparoscopic cholecystectomy (LC) without ERCP. NA: Not applicable.

by use of rendezvous technique (*P* = 0.24) (Table 3). According to the Cotton criteria<sup>[20]</sup>, one of these cases was classified as moderate and two as mild. Cannulation of the biliary duct was scored as difficult in nine (22%) cases where conventional ERCP was used, and precut sphincterotomy was performed in six of these (15%). There were three (7%) cases of unsuccessful cannulation and these failures were not associated with juxtapancreatic diverticula or other duodenal abnormalities. Contrast medium was inadvertently infused into the main pancreatic duct in 11 (27%) of the patients in the conventional ERCP group. In the rendezvous ERCP group, transcystic antegrade biliary rendezvous cannulation was successful in all 40 cases and no unintentional cannulation or opacification of the pancreatic duct occurred.

Complete CBDS clearance at the index procedure was achieved in 38 (95%) of the rendezvous ERCP procedures vs 29 (71%) patients in the conventional ERCP group (*P* = 0.01), even though there was no significant difference in stone size (*P* = 0.40) or procedure time (*P* = 0.11) between the two study groups. After an additional ERCP, all patients, including the three whose initial cannulation failed, had their bile ducts cleared of stones.

In the 41 control patients, no case of pancreatic duct opacification during cholangiography was noted, nor was acute pancreatitis or hyperamylasemia observed. Two major postoperative complications were recorded: one patient had postoperative intra-abdominal bleeding which did not need surgical intervention and one had postoperative bile leakage from the cystic duct, which was successfully treated by endoscopic placement of a temporary stent.

#### Laboratory markers

The post-procedural time course of pancreatic amylase showed no difference between the controls and the ren-

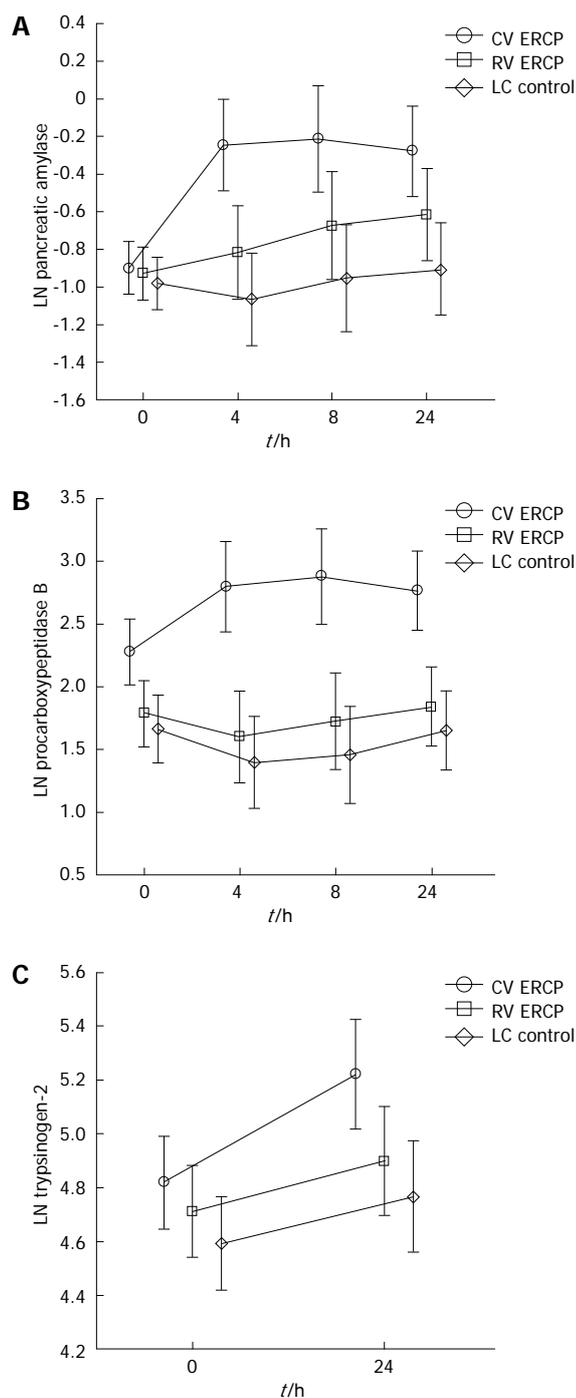
dezvous ERCP group (Figure 2A). In those subjected to a conventional ERCP, amylase levels had already increased significantly at 4 h and remained elevated throughout the 24-h monitoring time. Virtually the same picture emerged concerning the plasma levels of proCAPB (Figure 2B). Plasma concentrations of trypsinogen-2 were also significantly increased at 24 h in patients subjected to a conventional ERCP (Figure 2C). CRP values rose as expected in the groups subjected to surgery (rendezvous and control group) at 24 h after the completion of the procedure.

We explored other possible confounders but were unable to demonstrate any relationship between these laboratory variables and procedure time, the number of cannulation attempts, age, gender, comorbidity, degree of CBD dilatation, number or size of CBDS or the need for precut cannulation techniques. However, if side branches of the pancreatic duct side had been filled with contrast medium, this was significantly associated with the plasma level of proCAPB (*P* = 0.009) and most likely pancreatic amylase (*P* = 0.051).

## DISCUSSION

This study shows that transcystic guide wire assisted rendezvous cannulation at ERCP can be done in conjunction with bile duct stone clearance without increasing post-procedural leakage of pancreatic enzymes. This observation supports the hypothesis that rendezvous cannulation can prevent the risk of PEP. In fact, inadvertent contrast injection into the pancreatic duct during conventional ERCP was the factor most clearly associated with leakage of pancreatic enzymes into the systemic circulation, which we interpret as indicating risk of developing PEP.

The correlation between ERCP and PEP can be difficult to study, owing to the large number of patients required to ensure sufficient statistical power. In order to



**Figure 2** Approximate analysis and figure based on serum values at all times (0, 4, 8 and 24 h) using a mixed linear model. A: Pancreatic amylase before (0) and at 4, 8 and 24 h after the procedure; B: Procarboxypeptidase B before (0) and at 4, 8 and 24 h after the procedure; C: Trypsinogen-2 before (0) and at 4, 8 and 24 h after the procedure. y-axis values are logarithmically transformed. Vertical bars denote 95% confidence intervals. Circles: Conventional endoscopic retrograde cholangiopancreatography (ERCP); Squares: Rendezvous ERCP; Diamonds: Laparoscopic cholecystectomy control.

overcome these methodological difficulties, we utilised two biochemical markers, proCAPB and trypsinogen-2, as surrogate variables of pancreatic inflammatory response. These have both been shown to be superior to lipase and pancreatic amylase as diagnostic markers of acute pancreatitis, especially if the objective is to distin-

guish mild from severe pancreatitis<sup>[21,22]</sup>. ProCAPB and trypsinogen-2 have also been shown to discriminate pancreatitis from non-pancreatic disorders with an accuracy of 95% to 99%<sup>[19,21]</sup>. This distinction is essential, since two of our three study groups were subjected to additional surgical trauma in the form of laparoscopic cholecystectomy, which might increase unspecific inflammatory responses.

A potential weakness of the study is the lack of randomisation. Indeed, our original intention was to perform a randomised controlled trial. However, based on the promising results of our previous attempts with rendezvous ERCP<sup>[23]</sup>, we considered it ethically indefensible to perform conventional ERCP intraoperatively. First, the operating theatre during ongoing surgery is poorly suited for conventional ERCP and endoscopy under suboptimal circumstances would expose the patient to an unnecessary risk of pancreatic injury. Second, a study design where patients are randomised to postoperative ERCP could increase the risk of cannulation failures that would necessitate additional surgical exploration of the common bile duct. Therefore we considered this alternative study design more appropriate to address our research question. The present case-control design opens for selection bias, *e.g.*, in that patients selected for laparoscopic cholecystectomy with or without rendezvous ERCP were younger than those selected for conventional ERCP, of whom most had already their gallbladder removed, in some cases years earlier. However, this is to some degree counterbalanced by the fact that young age has repeatedly been found to be an independent risk factor for PEP<sup>[3,4,24,25]</sup> whereas older patients may be protected, *e.g.*, by age-related pancreatic atrophy<sup>[26]</sup>. These factors would be more likely to underestimate than overestimate the associations we found.

We observed only three cases of the clinically relevant outcome, PEP, all in the group subjected to conventional ERCP and none in the rendezvous group. This difference did not reach statistical significance, most likely due to low statistical power. Previous trials have proposed a relationship between rendezvous cannulation and a decreased risk of PEP but to date these studies have been either too small or not adequately designed to address this pertinent question. A recent systematic review by La Greca *et al.*<sup>[10]</sup> concluded that intraoperative ERCP and bile duct clearance during laparoscopic cholecystectomy was associated with low risk of PEP and could be recommended. Scrutiny of the cited papers reveals that in cases involving complete transcystic bile duct cannulation, where the guide wire was brought into the duodenal lumen, the incidence of PEP was even lower, ranging from 1.7% to 2.2%<sup>[27,28]</sup>. This contrasts to studies in which cannulation was not complete, where the corresponding incidence of PEP ranged from 3% to 7.6%<sup>[29-32]</sup>. Our data confirm to these previous findings and highlight the relevance of completing the rendezvous guide wire based cannulation to minimise PEP.

In most conventional ERCP procedures, biliary cannulation is easy, completely uneventful and antegrade

cannulation can be regarded as redundant. Our definition of difficult cannulation is arbitrary and subjective; however, even the most experienced endoscopist cannot predict whether a cannulation is going to be difficult or not until an attempt has been made, and each attempt at cannulation is potentially harmful. This is one of several arguments in favour of making rendezvous cannulation routine during laparoscopic cholecystectomy: regardless of the appearance of the papilla, the success rate of single-pass biliary cannulation approaches 100%. Repeat interventions, use of precut incision and so on should be kept to a minimum to diminish the risk of a variety of procedure-related complications.

Opacification of the pancreatic duct is another significant sign associated with difficult cannulation and the development of pancreatitis<sup>[3,8,24,25,33]</sup>. In our series, we found a significant correlation between elevated levels of amylase ( $P < 0.001$ ) or proCASP ( $P = 0.002$ ) and inadvertent cannulation and injection of contrast material into the pancreatic duct. Moreover, all three of our patients who developed PEP had had contrast medium injected into the pancreatic duct. Clearly, many of the risks associated with difficult cannulation, inadvertent pancreatic duct cannulation and contrast injection can be circumvented with rendezvous cannulation.

Current clinical evidence suggests that the risk of pancreatitis can be reduced, at least among high-risk patients, by temporary placement of a pancreatic stent that dislodges spontaneously<sup>[34,35]</sup>. At the time we began our study there was no routine of using prophylactic pancreatic stents at our institution and we have therefore not offered this option during the study. Such stenting could clearly have affected the outcome for patients who were allocated to the conventional ERCP group and had a guidewire or minimal contrast injection into the pancreatic duct. Nonetheless, we firmly believe that it is preferable to use a technique that completely avoids cannulation of the pancreatic duct.

Transient asymptomatic hyperamylasemia has very little clinical significance and is usually not recognised as a complication. In the present study the levels of amylase after conventional ERCP were increased at 4, 8 and 24 h compared with levels in the rendezvous and the control group. These results are in line with other reports<sup>[36]</sup>, where a majority of the ERCP procedures involving cannulations that were characterised as difficult were associated with hyperamylasemia and PEP.

A few prospective studies<sup>[16,18,37]</sup> demonstrate that patients affected by PEP have significantly elevated concentrations of trypsinogen-2 in urine six and 24 h after the index procedure, even in if the pancreatitis is mild. Our study confirms these findings: we found higher concentrations of trypsinogen-2 in plasma at 24 h in the conventional ERCP group than in the rendezvous and control groups. Taken together the surrogate markers we have used to detect early signs of PEP appear to be of high clinical relevance.

An additional advantage of the rendezvous approach, one of utmost clinical relevance, appeared when we ana-

lysed the CBDS clearance rate. Even though all stones were eventually cleared in both study groups, there was a significant advantage for the rendezvous technique during the index procedure. This finding is in line with the compilation of 21 reports by La Greca *et al*<sup>[10]</sup> which demonstrated an overall success rate of 92.3% for rendezvous ERCP.

In conclusion, combined laparo-endoscopic trans-cystic guidewire rendezvous cannulation of the CBD minimizes the risk of unintentional pancreatic duct engagement and subsequent inflammatory damage to the gland. In addition, more complete CBDS removal can be offered. Thus, intraoperative ERCP with rendezvous cannulation technique shall be recommended to manage CBDS in conjunction with cholecystectomy.

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## COMMENTS

### Background

Endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy and stone extraction is a standard procedure in the management of common bile duct stones (CBDS). The method is, however, marred by complications like pancreatitis, bleeding and perforation. Intraoperative rendezvous ERCP during cholecystectomy with cannulation over a guidewire represents an alternative method to remove CBDS that theoretically may, due to a significantly reduced risk to cannulate the pancreatic duct, decrease the risk of procedural induced pancreatitis.

### Research frontiers

The concept of rendezvous cannulation involving a combined laparo-endoscopic approach and used in conjunction with intraoperative ERCP has been described previously. The question whether intraoperative rendezvous ERCP reduces the risk of post ERCP pancreatitis (PEP) rate or not is, however, not known mainly due to the fact that the incidence of PEP is rather low and large study groups are therefore needed in order to reach enough statistical power to investigate the impact of different ERCP techniques.

### Innovations and breakthroughs

To overcome the methodological difficulties in studying the effects of intraoperative rendezvous ERCP on PEP the authors utilised two highly sensitive and specific biochemical markers, procarboxypeptidase B and trypsinogen-2, as surrogate variables of pancreatic inflammatory response. These have both been shown to be superior to lipase and pancreatic amylase as diagnostic markers of acute pancreatitis, especially if the objective is to distinguish mild from severe pancreatitis.

### Applications

The study results have helped to obtain an increased knowledge of the beneficial effects that intraoperative rendezvous ERCP may have in reducing PEP. The proposed mechanism for this is that, due to cannulation over the guidewire, the risk of cannulating the pancreatic duct and thus injecting contrast in the duct is minimal.

## Terminology

ERCP: An endoscopic method to examine the bile- and pancreatic ducts as well as to, by intervention, remove, common bile duct stones or relieve bile duct obstruction. Pancreatic amylase, procarboxypeptidase B and trypsinogen-2: Biochemical markers that serve as surrogate variables of pancreatic inflammatory response.

## Peer review

The authors of this paper report that intraoperative rendezvous ERCP reduces the release of the pancreatic markers (pancreatic amylase, procarboxypeptidase B and trypsinogen-2) compared with conventional ERCP. As only a few papers have been published in this topic, this paper can provide additional data for the accumulation of the knowledge in this field. The case control design of the study, however, opens for selection bias which weakens the power of the conclusion.

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## Outcomes of Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding

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### Abstract

**AIM:** To evaluate weight loss and surgical outcomes of Roux-en-Y gastric bypass (RYGB) and laparoscopic adjustable gastric band (LAGB).

**METHODS:** Data relating to changes in body mass index (BMI) and procedural complications after RYGB (1995-2009;  $n = 609$ ; 116M: 493F;  $42.4 \pm 0.4$  years) or LAGB (2004-2009;  $n = 686$ ; 131M: 555F;  $37.2 \pm 0.4$

years) were extracted from prospective databases.

**RESULTS:** Pre-operative BMI was higher in RYGB than LAGB patients ( $46.8 \pm 7.1$  kg/m<sup>2</sup> vs  $40.4 \pm 4.2$  kg/m<sup>2</sup>,  $P < 0.001$ ); more patients with BMI  $< 35$  kg/m<sup>2</sup> underwent LAGB than RYGB (17.1% vs 4.1%,  $P < 0.0001$ ). BMI decrease was greater after RYGB. There were direct relationships between weight loss and pre-operative BMI ( $P < 0.001$ ). Although there was no difference in weight loss between genders during the first 3-year post-surgery, male LAGB patients had greater BMI reduction than females ( $-8.2 \pm 4.3$  kg/m<sup>2</sup> vs  $-3.9 \pm 1.9$  kg/m<sup>2</sup>,  $P = 0.02$ ). Peri-operative complications occurred more frequently following RYGB than LAGB (8.0% vs 0.5%,  $P < 0.001$ ); majority related to wound infection. LAGB had more long-term complications requiring corrective procedures than RYGB (8.9% vs 2.1%,  $P < 0.001$ ). Conversion to RYGB resulted in greater BMI reduction ( $-9.5 \pm 3.8$  kg/m<sup>2</sup>) compared to removal and replacement of the band ( $-6.0 \pm 3.0$  kg/m<sup>2</sup>). Twelve months post-surgery, fasting glucose, total cholesterol and low density lipoprotein levels were significantly lower with the magnitude of reduction greater in RYGB patients.

**CONCLUSION:** RYGB produces substantially greater weight loss than LAGB. Whilst peri-operative complications are greater after RYGB, long-term complication rate is higher following LAGB.

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**Key words:** Bariatric surgery; Gastric bypass; Gastric banding; Weight loss; Complications; Co-morbidity; Outcomes

**Core tip:** Roux-en-Y gastric bypass (RYGB) produces substantially greater weight loss and resolution of co-morbidities than laparoscopic adjustable gastric band (LAGB) in a community setting, in both the short-

and long-term. Although peri-operative complications are higher with RYGB than LAGB, which are non-fatal and mostly related to wound infection, the long-term complication rate is higher after LAGB. Where LAGB fails to induce or maintain weight loss, RYGB appears to be the superior salvage procedure. The better outcomes for LAGB in males compared to females after 3 years post-surgery are intriguing and needs further confirmation.

Nguyen NQ, Game P, Bessell J, Debreceni TL, Neo M, Burgstad CM, Taylor P, Wittert GA. Outcomes of Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding. *World J Gastroenterol* 2013; 19(36): 6035-6043 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6035.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6035>

## INTRODUCTION

Obesity is increasing in prevalence in the Western world and affects approximately 30% of the population<sup>[1,2]</sup>. It is associated with significant co-morbidities including diabetes, cardiovascular disease and obstructive sleep apnoea<sup>[1,3]</sup>. Unfortunately, medical treatment with lifestyle modification or pharmacotherapy is only modestly effective with a high relapse rate<sup>[4,5]</sup>. Bariatric surgery is recommended for patients with a body mass index (BMI) > 40 kg/m<sup>2</sup> or BMI > 35 kg/m<sup>2</sup> with significant co-morbidities<sup>[2,3]</sup>. Two commonly performed bariatric procedures are Roux-en-Y gastric bypass (RYGB) and laparoscopic gastric banding (LAGB)<sup>[2,3]</sup>.

Currently, the choice between these bariatric procedures is based mainly on patient and surgeon preference, and varies significantly between regions of the world. In contrast to the United States and Europe, LAGB is the most common procedure in Australia (90%; with -10% RYGB)<sup>[6,7]</sup>, perhaps because LAGB is perceived as a safer, minimally invasive, fully reversible and adjustable procedure<sup>[6,7]</sup>. In Australia, the number of LAGB procedures increased by 10 times over the last decade, as compared to the stable rate of RYGB procedures (Figure 1). This is despite the majority of available data, including the recent systematic and network meta-analysis of randomised trials on weight loss outcome at 1 year, indicating that RYGB produces greater weight loss with more frequent resolution of type 2 diabetes, hypertension, dyslipidemia and sleep apnoea (OSA)<sup>[3,8-10]</sup>.

For LAGB, meticulous follow up has been suggested to play an important role in achieving and maintaining the weight loss, and this may be responsible for some of the impressive weight loss reported by Australian LAGB centers<sup>[7,11-13]</sup>. Alternatively drop-outs or “treatment failures” may be under-reported. Thus, the aim of the current study was to compare weight loss and surgical outcomes of RYGB and LAGB from two large, prospectively maintained surgical databases.

## MATERIALS AND METHODS

Data from two clinical databases of obese patients who underwent primary RYGB or LAGB were reviewed. The data have been collected prospectively and maintained by two experienced bariatric surgeons performing predominantly either RYGB (PG) or LAGB (JB) in Adelaide, South Australia. The RYGB cohort included all patients who had surgery from 1995 to 2009, by either open or laparoscopic techniques. The LAGB group included all patients who underwent the procedure from 2004 to 2009 (Figure 2). The RYGB cohort included both public and private patients, whereas all patients in the LAGB group were treated in private hospitals.

### Patient selection

For both RYGB and LAGB cohorts, the patients were assessed individually by the respective surgeon and a dietician as well as, in most cases, a multi-disciplinary team (physician, exercise physiologist, psychologist) prior to surgery. In general, the decision to undertake bariatric surgery followed the recommendations of the American Society for Metabolic and Bariatric Surgery Clinical Issues Committee<sup>[1]</sup>.

### Data collection

Both databases were kept electronically (Microsoft Office Access<sup>®</sup> for RYGB cohort and Filemaker Pro V8 for LAGB cohort) and maintained by the surgeon and the practices. For all patients, information on demographics, pre-operative and post-operative body mass index, and pre-operative co-morbidities were recorded. Details on the peri-operative and long-term complications were also recorded for the affected cases. Scanned copies of serial laboratory measurements (fasting glucose, lipid profile and liver function test) were available in approximately 50% of cases.

Data on patient demographics, date and type of procedure and changes in BMI over the period of follow up were exported to excel files from Microsoft Office Access<sup>®</sup>. Data regarding obesity related co-morbidities, medications, resolution of co-morbidities, and serial laboratory measurements were collected by reviewing each patient’s files (both paper and electronic). Complete serial data on blood glucose, lipid profiles and changes on co-morbidities over the first 12 mo after surgery were available for 301 RYGB and 545 LAGB patients.

### Surgical techniques

**Open RYGB procedure:** After midline incision, a gastric pouch of approximately 25 mL was created by stapling off the proximal stomach with a TA 90B four-row stapler (Autosuture<sup>®</sup>, Covidien<sup>®</sup>), or the stomach was divided using a linear stapler (GIATM, Covidien<sup>®</sup> or TLCTM, Ethicon<sup>®</sup>) stapler. The pouch volume was not measured using upper gastrointestinal endoscopy or a balloon device. A retrocolic and retrogastric 100-cm Roux limb was

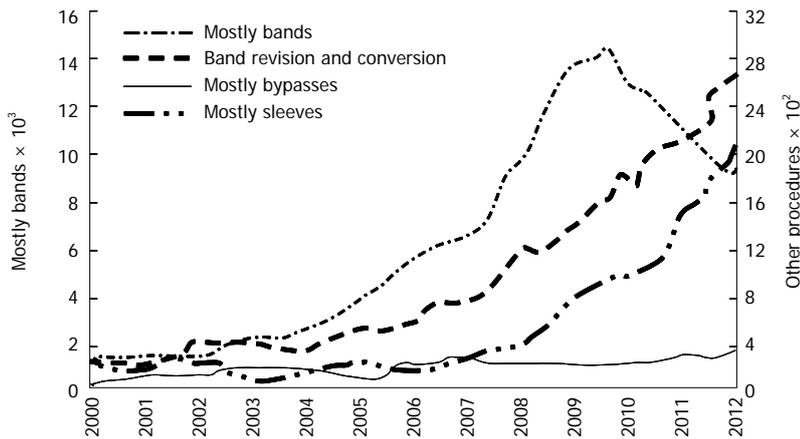


Figure 1 Comparison of changes in the performance of different type of bariatric procedures over a 10-year period in Australia based on medical benefit schedule item number.

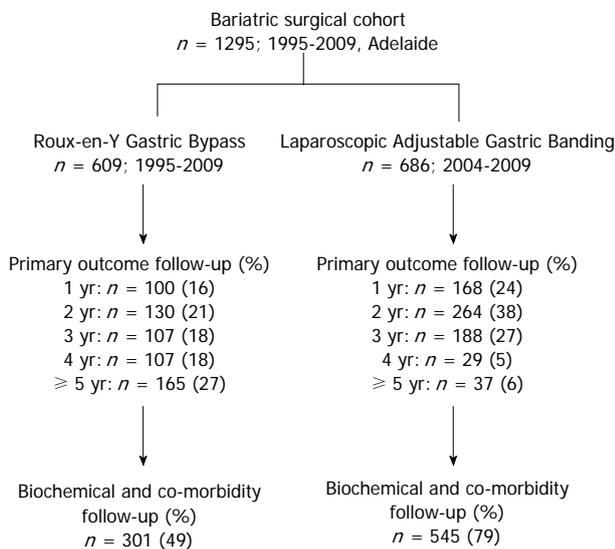


Figure 2 Flow chart of studied cohort in relation to clinical outcomes.

anastomosed to the gastric pouch using a two-layered hand-sewn technique, with an outer 3/0 polypropylene non-absorbable continuous layer and an inner 3/0 PDS absorbable continuous suture. A hand-sewn side-to-side jejuno-jejunal anastomosis was performed. The integrity of the gastro-jejunosomy was tested with methylene blue.

**Laparoscopic RYGB procedure:** A five-port technique was used. The stomach was transected with a laparoscopic linear stapler (Endo-GIATM, Covidien® or EndopathTM, Ethicon®). The two anastomoses were either performed with a hand-sewn technique or with stapling devices (EEATM OrViITM XL circular stapler, Endo-GIATM, Covidien®). Again, the integrity of the gastro-jejunosomy was tested with methylene blue.

**Laparoscopic adjustable gastric banding procedure:** Commercially available laparoscopic adjustable gastric

bands from Allergan Inc (Lap Band; Allergan, Inc, Irvine, California) were used. The size of the gastric band system used was at the discretion of the surgeon. The band was inserted using the pars flaccida technique, which involved adequate exposure of the angle of His by retracting the gastric fundus inferiorly. Dissection was continued until the left crus of the diaphragm was completely exposed. A small incision in the avascular aspect of the gastro-hepatic ligament was created, and care was taken to identify and preserve the hepatic branch of the vagus nerve. Blunt dissection was used to create a space between the base of the right crus and its overlying peritoneum. A long grasper was then gently passed above the right crus, underneath the gastroesophageal junction, toward the angle of His. The lap-band was passed around the gastroesophageal junction and snapped in place and secured with 3 gastrogastic sutures. The band reservoir was filled with 3 mL of normal saline for APS bands, and 4 mL of normal saline for APL bands.

**Post-operative care and follow up**

Patients from either centre followed a strict post-operative protocol. For both procedures, clear liquids were provided on the first post-operative day and a full-liquid diet on the second.

Patients undergoing RYGB were seen in the clinic 2 wk post-operatively, every 3 mo for the first year, and then annually thereafter. After LAGB, patients were asked to continue seeing a dietician, psychologist, exercise physiologist and a general practitioner with an interest in obesity and bariatric surgery. Most patients returned every 3 mo for the first year, and 6-monthly thereafter for patients who progressed well. Some patients may have seen the bariatric GP more frequently depending on progress and problems experienced. Band adjustments were most commonly performed at week 2 and 6 postoperatively, then every 3 mo for the first year (total of 5 visits). Adjustments to the band were performed according to manufacturer’s guidelines.

**Table 1** Characteristics of patients with Roux-en-Y gastric bypass and laparoscopic adjustable gastric band *n* (%)

	<b>RYGB (<i>n</i> = 609)</b>	<b>LAGB (<i>n</i> = 686)</b>
Gender (M:F)	116: 493	131: 555
Age (yr)	42.4 ± 10.5 <sup>b</sup>	37.2 ± 9.4
Initial BMI (kg/m <sup>2</sup> )	46.8 ± 7.1 <sup>b</sup>	40.4 ± 4.2
Co-morbidities (sub-group analysis)	( <i>n</i> = 301)	( <i>n</i> = 545)
Total co-morbidities	216 (71.0)	363 (66.6)
Type 2 diabetes mellitus ( <i>n</i> )	82	95
Hypertension ( <i>n</i> )	51	157
Hyperlipidemia ( <i>n</i> )	116	117
Obstructive sleep apnoea ( <i>n</i> )	97 <sup>b</sup>	113
Duration of follow-Median (yr)	2.0 (1.0-5.0)	1.6 (1.0-2.2)

<sup>b</sup>*P* < 0.01 vs LAGB. BMI: Body mass index; RYGB: Roux-en-Y gastric bypass; LAGB: Laparoscopic adjustable gastric band; M: Male; F: Female.

For both surgeries, most patients were advised to take supplementary vitamins (including vitamin D) and calcium.

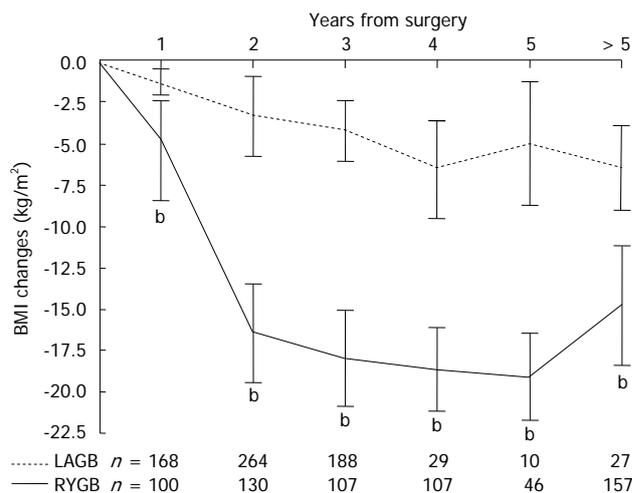
**Definitions of outcome measures**

**Primary outcome measures:** (1) Weight loss. This was expressed as change in BMI from baseline over the study duration; and (2) Complications and need for re-operation. Acute (< 30 d) and long-term (≥ 30 d) complications included use of unexpected drug therapy or imaging, total parenteral nutrition, a bedside procedure, blood transfusion, a hospital stay longer than twice the median stay, diagnostic or therapeutic endoscopy, re-operation (with or without organ resection or anastomotic revision), or death.

**Secondary outcome measures:** (1) Diabetes mellitus and hyperlipidemia. As data regarding the need for anti-diabetic or anti-cholesterol medications were incomplete, only improvement rather than “resolution” of diabetes mellitus or hyperlipidemia could be assessed, and the change from baseline to 12 mo was compared between the procedures. Improvement in diabetes mellitus was defined as a fasting blood glucose < 5.5 mmol/L. Improvement in hyperlipidemia was expressed as a percentage of patients who had normalization of plasma total cholesterol (level < 5.5 mmol/L) or plasma triglycerides (level < 2.0 mmol/L); and (2) Obstructive sleep apnoea. Resolution of obstructive sleep apnea was defined as the absence of requirement for continuous positive airway pressure after surgery.

**Statistical analysis**

Data were expressed as mean ± SD, unless stated otherwise. Comparison of variables between the two surgical groups was undertaken using  $\chi^2$  tests for categorical data and independent t-test for continuous data sets. The differences in changes in weight loss outcome over time between the groups were compared using Kaplan Meier analysis. All analyses were performed using GraphPad Prism statistical software, version 6 (GraphPad Software



**Figure 3** Changes in body mass index in patients who underwent Roux-en-Y gastric bypass and laparoscopic adjustable gastric band up to 5 years post-surgery. <sup>b</sup>*P* < 0.001 vs laparoscopic adjustable gastric band (LAGB). RYGB: Roux-en-Y gastric bypass.

Inc., La Jolla, CA, United States).

**RESULTS**

A total of 1295 patients was included in the study; 609 underwent RYGB (116M:493F; age: 42.4 ± 10.5 years) and 686 underwent LAGB (131M:555F; age: 37.2 ± 9.4 years). For the RYGB cohort, 13% (78) of procedures were performed laparoscopically. The initial BMI was significantly higher in patients who underwent RYGB than LAGB (46.8 ± 7.1 kg/m<sup>2</sup> vs 40.4 ± 4.2 kg/m<sup>2</sup>, *P* < 0.01). Overall, 161/1295 (12%) patients underwent bariatric surgery with an initial BMI between 30 and 35 kg/m<sup>2</sup>, and it was more prevalent in patients who underwent LAGB (128/686 vs 33/609, *P* < 0.0001). The median number of follow up visits after surgery was 6 in both groups, with 63% of RYGB patients and 38% of LAGB patients had follow-up duration of greater than 3 years (Table 1 and Figure 2).

**Body mass index**

Overall, RYGB resulted in a significantly greater decrease in BMI than LAGB (-14.8 ± 3.4 kg/m<sup>2</sup> vs -2.9 ± 1.2 kg/m<sup>2</sup>, *P* < 0.0001). The greater reduction in BMI after RYGB over LAGB was observed at all time points, and the peak weight loss was observed at year 4 for both procedures (Figure 3). Irrespective of pre-operative BMI, weight reduction was greater after RYGB than LAGB (*P* < 0.001, Figure 4). For both surgical groups, there was a direct relationship between weight loss and the pre-operative BMI (*P* < 0.001) (Figure 4).

**Complication and re-operation rates**

Peri-operative complications were higher with RYGB than LAGB (8.0% vs 0.5%, *P* < 0.001). The majority of the acute complications in the RYGB group were minor and did not require any surgical intervention (Table 2). Long-

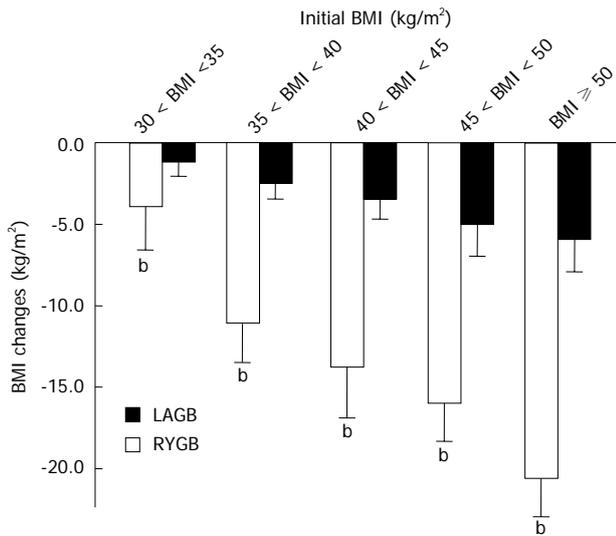


Figure 4 Relationship between the degree of weight loss and presenting body mass index induce by Roux-en-Y gastric bypass and laparoscopic adjustable gastric band procedure. <sup>b</sup>*P* < 0.001 vs laparoscopic adjustable gastric band (LAGB). RYGB: Roux-en-Y gastric bypass; BMI: Body mass index.

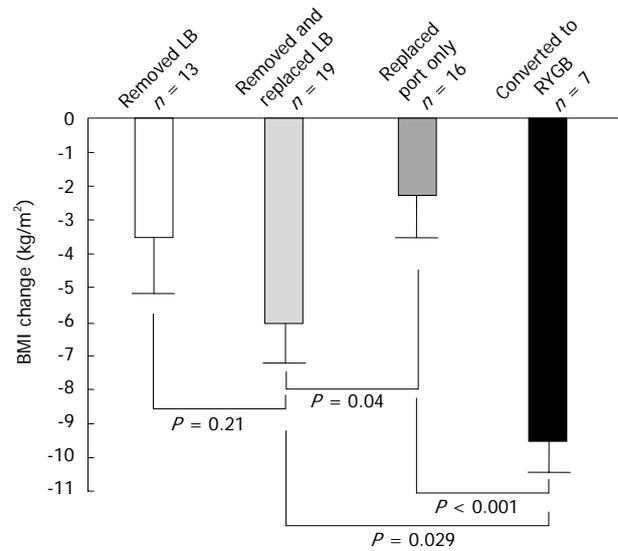


Figure 5 Outcomes of patients who had complications with laparoscopic adjustable gastric band requiring further interventions, including removal of band, removal and placement of band, replacement of port only and conversion to Roux-en-Y gastric bypass. RYGB: Roux-en-Y gastric bypass; LB: Lap-band; BMI: Body mass index.

Table 2 Acute- and long-term complications between Roux-en-Y gastric bypass and laparoscopic adjustable gastric band	
RYGB (n = 609)	LAGB (n = 686)
Acute complications (n)	
n = 49 (8.0%)	n = 3 (0.5%) ( <i>P</i> < 0.0001 vs RYGB)
Wound infection (35)	Small leak, required band removal after 1 d (1)
Abdominal sepsis (3)	Post-op respiratory infection (1)
Splenic trauma (2)	Large wound haematoma, drained spontaneously (1)
DVT and PE (2)	
Endoscopy for stomal obstruction (4)	
Mechanical failure (3)	
Long-term complications (n)	
n = 13 (2.1%)	n = 61 (8.9%) ( <i>P</i> < 0.0001 vs RYGB)
Reversal of RYGB (n = 1)	Removed and replaced LB (19)
Incisional hernia (n = 7)	Replacement of port (16)
Bowel obstruction (n = 5)	Removed LB (13)
	Converted to RYGB (7)
	Stomal obstruction required dilation (3)
	Mechanical failure (3)

RYGB: Roux-en-Y gastric bypass; LAGB: Laparoscopic adjustable gastric band; LB: Lap-band.

term complications necessitating corrective procedures were higher following LAGB than RYGB (8.9% vs 2.1%, *P* < 0.001). Conversion to RYGB resulted in a greater BMI reduction ( $-9.5 \pm 3.8 \text{ kg/m}^2$ ) as compared to removal and replacement of the band ( $-6.0 \pm 3.0 \text{ kg/m}^2$ ) (Figure 5).

**Impact of gender on outcomes of RYGB and LAGB**

Pre-operative BMI was similar in males and females undergoing LAGB ( $41.1 \pm 4.5$  vs  $40.3 \pm 4.3 \text{ kg/m}^2$ ), but greater in males than females undergoing RYGB ( $47.9 \pm 4.9$  vs  $44.9 \pm 3.8 \text{ kg/m}^2$ , *P* < 0.01). RYGB induced greater weight loss than LAGB in both genders (male:

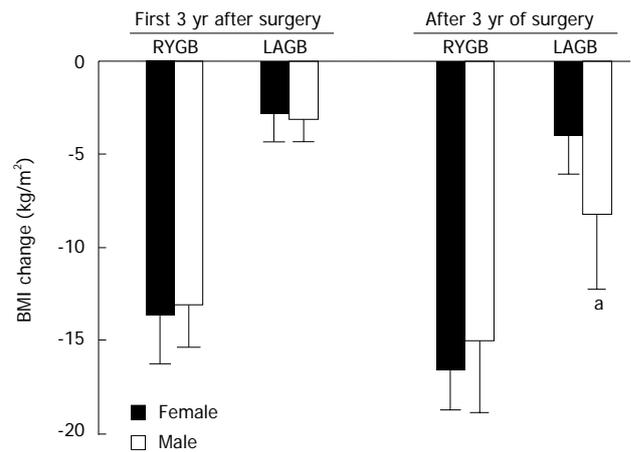


Figure 6 Body mass index changes after Roux-en-Y gastric bypass and laparoscopic adjustable gastric band in male and female 3 years post-surgery. <sup>a</sup>*P* = 0.02 vs female. RYGB: Roux-en-Y gastric bypass; LAGB: Laparoscopic adjustable gastric band.

$-13.8 \pm 3.1$  vs  $-3.5 \pm 1.9 \text{ kg/m}^2$ ; *P* < 0.0001; female:  $-15.0 \pm 3.8$  vs  $-2.9 \pm 1.3 \text{ kg/m}^2$ , *P* < 0.0001), with no differences in weight loss between genders during the first 3 years of surgery. Thereafter LAGB males had a greater reduction in BMI than females ( $-8.2 \pm 4.3$  vs  $-3.9 \pm 1.9 \text{ kg/m}^2$ , *P* = 0.02, Figure 6).

The rates of acute (male: 9/116 vs female: 40/493) or long-term complications (male: 2/116 vs female: 11/493) were similar in males and females after RYGB. In the LAGB cohort, however, longer term complications requiring corrective procedures, were less likely to occur in males than females (male: 2/131 vs female: 59/555, *P* < 0.001). The rate of acute complication for both genders was similar after LAGB (male: 0/131 vs female: 4/555).

**Table 3 Characteristics of patients with and without biochemical data**

	RYGB (n = 609)			LAGB (n = 686)		
	Biochemical data (n = 301)	No biochemical data (n = 308)	P-value	Biochemical data (n = 545)	No biochemical data (n = 141)	P-value
Gender (M:F)	63:238	53:255	> 0.05	101:444	30:111	> 0.05
Age (yr)	42.9 ± 10.2	42.0 ± 10.6	> 0.05	37.8 ± 9.4	37.1 ± 9.5	> 0.05
Initial BMI (kg/m <sup>2</sup> )	45.9 ± 7.5	47.0 ± 7.4	> 0.05	40.6 ± 4.1	40.3 ± 4.2	> 0.05
BMI reduction at 12 mo (kg/m <sup>2</sup> )	-6.5 ± 3.4	-7.7 ± 4.6	> 0.05	-1.2 ± 0.8	-1.4 ± 0.9	> 0.05
BMI reduction at 3 yr (kg/m <sup>2</sup> )	-18.2 ± 3.9	-19.0 ± 4.9	> 0.05	-4.9 ± 2.6	4.3 ± 2.4	> 0.05

BMI: Body mass index; RYGB: Roux-en-Y gastric bypass; LAGB: Laparoscopic adjustable gastric band; M: Male; F: Female.

**Table 4 Co-morbidities before and 12 mo after Roux-en-Y gastric bypass and laparoscopic adjustable gastric band n (%)**

	RYGB (n = 301)	LAGB (n = 554)	P-value (RYGB vs LAGB)
Diabetes mellitus			
Pre-operative	83 (28)	127 (23)	0.34
Normalization of fasting blood glucose <sup>1</sup>	26 (33)	22 (17)	0.02
Hyper-cholesterolemia			
Pre-operative	125 (42)	224 (41)	0.77
Normalization of total plasma cholesterol <sup>1</sup>	65 (52)	9 (4)	< 0.001
Hyper-triglyceridemia			
Pre-operative	63 (21)	124 (22)	0.86
Normalization of plasma triglyceride <sup>1</sup>	51 (81)	34 (27)	< 0.0001
Obstructive sleep apnoea			
Pre-operative	100 (33)	130 (23)	0.02
No longer required CPAP at 12 mo	10 (10)	4 (3)	0.04

<sup>1</sup>Compared to pre-operative. RYGB: Roux-en-Y gastric bypass; LAGB: Laparoscopic adjustable gastric band; CPAP: Continuous positive airway pressure.

**Table 5 Fasting blood glucose and lipid profile after Roux-en-Y gastric bypass and laparoscopic adjustable gastric band**

	RYGB (n = 301)	LAGB (n = 554)	P-value (RYGB vs LAGB)
Fasting blood sugar			
Prior to surgery	5.9 ± 1.6	5.7 ± 1.7	0.12
6-month post-op	5.3 ± 1.6 <sup>b</sup>	5.5 ± 1.9 <sup>b</sup>	0.57
12-month post-op	5.0 ± 1.7 <sup>b</sup>	5.4 ± 1.6 <sup>b</sup>	0.02
Total cholesterol			
Prior to surgery	5.2 ± 1.0	5.2 ± 1.0	0.65
6-month post-op	4.2 ± 0.9 <sup>b</sup>	5.2 ± 1.0	< 0.0001
12-month post-op	4.3 ± 0.9 <sup>b</sup>	5.0 ± 1.0 <sup>b</sup>	< 0.0001
Total triglyceride			
Prior to surgery	1.7 ± 0.5	2.0 ± 0.6	0.44
6-month post-op	1.2 ± 0.6 <sup>b</sup>	1.5 ± 0.6	0.01
12-month post-op	1.1 ± 0.4 <sup>b</sup>	1.5 ± 0.5	0.04
HDL			
Prior to surgery	1.3 ± 0.3	1.3 ± 0.3	0.26
6-month post-op	1.3 ± 0.3	1.4 ± 0.3 <sup>b</sup>	0.21
12-month post-op	1.4 ± 0.3 <sup>b</sup>	1.5 ± 0.3 <sup>b</sup>	0.56

<sup>b</sup>P < 0.01 vs baseline. RYGB: Roux-en-Y gastric bypass; LAGB: Laparoscopic adjustable gastric band; HDL: High-density lipoprotein; op: Operative.

**Blood sugar, lipid profiles and co morbidities**

There were no differences in age, gender, initial BMI and magnitude of weight loss between those patients for whom biochemical/co-morbidity data was, or was not, available (Table 3). Before surgery, there were no differences in the proportion of patients with diabetes mellitus, hyperlipidemia or hypertension (Table 1). The presence of significant co-morbidities (diabetes mellitus, hypertension, sleep apnoea) in patients who had initial BMI between 30-35 kg/m<sup>2</sup> was 45% in both RYGB and LAGB groups.

Fasting blood glucose (33% vs 17%, P = 0.02), total cholesterol (54% vs 4%, P < 0.001), and plasma triglyceride (81% vs 27%, P < 0.0001) normalised more frequently after RYGB than after LAGB, respectively (Table 4). Fasting blood glucose, total cholesterol and triglyceride levels were significantly reduced 12 mo after both types of procedures, however the magnitude of improvement was greater and the onset of improvement was earlier after RYGB (Table 5). Both procedures resulted in a similar increase of plasma high-density lipoprotein.

Patients who underwent RYGB were more likely to have obstructive sleep apnea (OSA: 32% vs 20%, P < 0.001; Table 3). The proportion of patients who had resolution of OSA were significantly higher after RYGB than after LAGB (10% vs 3%, P = 0.03; Table 4).

**DISCUSSION**

For patients who underwent bariatric surgery for morbid obesity this data shows that, while both procedures are safe, RYGB was associated with: (1) substantially greater weight loss; (2) lower fasting blood glucose, total cholesterol and triglyceride levels; (3) greater risk of acute non-fatal complications; and (4) lower rate of re-operation rate in long-term. Following band failure, conversion to RYGB resulted in a greater reduction in BMI, than band replacement, and may therefore be a preferable rescue procedure. This may be particularly relevant for patients with inadequate resolution of co-morbidities after LAGB. Furthermore, the current study demonstrates the differential impact of gender on weight loss and complications in patients who underwent LAGB but not RYGB. The greater weight loss observed in males 3

years post lap band insertion was associated with a significantly lower rate of long-term band related complications.

The findings of this study are consistent with the available data from both randomized and non-randomized clinical trials<sup>[3,8-10,14-18]</sup>. Currently, only 2 prospective randomized comparisons of RYGB against LAGB have been performed and both have methodological weaknesses<sup>[8,17]</sup>. Although the first study found better short-term weight loss and a lower number of weight loss failures after RYGB, the sample size was small (24 RYGB *vs* 27 LAGB) limiting the capacity to make definitive conclusions, particularly about uncommon adverse events<sup>[17]</sup>. The larger, second trial (111 RYGB *vs* 86 LAGB) confirmed the superior weight loss after RYGB as compared to LAGB, but was criticized for significant differences in baseline BMI between the groups and an unusual high rate of gastro-jejunostomy stricture<sup>[8]</sup>. Both studies showed a higher rate of peri-operative but lower incidence of later complications and need for re-operation after RYGB than LAGB<sup>[8,17]</sup>. Similar outcomes have been reported from pair-matched studies, as well as systematic and network meta-analyses<sup>[3,8-10,14-18]</sup>.

The marked improvement or normalization of fasting blood glucose and lipid profile after both RYGB and LAGB in this current study are in keeping with previous studies<sup>[3,8-10,14-18]</sup>. The observation that, of the two procedures, RYGB has a much greater benefit for these co-morbidities, is consistent with previously reported data<sup>[3,9]</sup>. The greater proportion of patients with OSA in the RYGB group is most likely related to the greater initial BMI, which is a known risk factor for OSA. Similar, the greater resolution of OSA after RYGB, as compared to LAGB, is most likely related to the greater reduction in BMI, and the finding is consistent with the current literature<sup>[3]</sup>.

The differences in acute and long-term complications in the current study are also in keeping with the current literature<sup>[3,8-10,14-18]</sup>. Given RYGB is a more complex operation with a longer operative time, it is not surprising that the prevalence of acute post-operative complications was higher after RYGB than LAGB. It is, however, important to note that none of the complications were fatal and most were related to wound infection and managed successfully with medical therapy. Compared to the 1% mortality reported internationally, there were no surgical deaths amongst our RYGB cohort. The reasons for the absence of mortality may relate to the meticulous patient assessment and selection, pre-operative preparation, and anaesthetist with a special interest and expertise in dealing with complex bariatric patients. The impressively low rate of acute complications after LAGB in this report should be highlighted and is consistent with the current literature<sup>[3,8-10,14-18]</sup>, suggesting that LAGB is an extremely safe bariatric procedure.

Except for the study of Nguyen *et al*<sup>[8]</sup>, which was criticized for the unusually high rate of gastro-jejunos-

tomy stricture after RYGB, our and other long-term follow-up studies have consistently shown a higher rate of long-term complication and the need for re-operation after LAGB as compared to RYGB<sup>[3,8-9]</sup>.

The superior weight loss at 3 years after LAGB in males as compared with females may relate, at least in part, to a lower number of postoperative long-term complications. Other studies have either shown similar<sup>[14,19,20]</sup> or worse outcomes<sup>[8,21]</sup> for males than females after LAGB. One reason for this is the poor adherence of men to post-band advice and follow-up<sup>[22,23]</sup>. On the other hand, men are less likely to have over-eating disorders, are more willing to exercise if feeling well<sup>[22,23]</sup>, and generally have better outcomes in most studies of diet induced weight loss<sup>[24]</sup>. In the most recent large study of LAGB procedures ( $n = 3000$ ), the occurrence of proximal gastric pouch dilatation, a known risk factor for poor weight loss, was significantly more common in women than men (5.1% *vs* 1.3%), and was highest in younger women<sup>[23]</sup>. The possibility of selection biases by selectively chosen highly motivated males cannot be excluded. Regardless, better outcomes for LAGB in males than females indicates that gender may be an important consideration in procedure choice and needs further confirmation.

It is notable that about 12% of patients with BMI between 30 and 35 kg/m<sup>2</sup> underwent bariatric surgery, particularly in the LAGB group (18% *vs* 5% in RYGB group). This may reflect patient pressure in the private system, concomitant co-morbidities or pre-operative weight loss prior to the initial consultation, together with the perception that LAGB is reversible and safe. Weight loss in this group of patients was particularly poor (RYGB:  $-3.9 \pm 0.3$  kg/m<sup>2</sup>, and LAGB:  $-1.5 \pm 0.3$  kg/m<sup>2</sup>), highlighting the rigorous protocols for patient selection in order to optimise outcomes.

There are strengths and weaknesses in this current study. The large sample size, a well maintained prospective database and an extended follow-up are the strengths of the current study. To our knowledge, our study is the largest comparative study on RYGB versus LAGB with follow up over 5 years. On the other hand, the weaknesses are potential selection biases by 2 independent surgeons who only performed either RYGB or LAGB, incomplete data relating to plasma biochemistry, and the lack of details on medications for co-morbidities.

In a community setting, RYGB produces substantially greater weight loss and resolution of co-morbidities than LAGB in both the short- and long-term, at a cost of higher peri-operative complications, which are non-fatal and mostly related to wound infection. The long-term complication rate is higher after LAGB. Where band failure occurs, RYGB is the superior salvage procedure. The better outcomes for LAGB in males compared to females after 3 years post-surgery needs further confirmation. Gender, like extent of co-morbidities and

BMI, may be an important consideration in procedure choice.

## COMMENTS

### Background

Currently, the choice between these bariatric procedures is based mainly on patient and surgeon preference, and varies significantly between regions of the world. In contrast to the United States and Europe, laparoscopic adjustable gastric band (LAGB) is the most common procedure in Australia and the number of procedures increased by 10 times over the last decade. This is despite the majority of available data indicating that LAGB produces smaller weight loss and less frequent resolution of comorbidities than Roux-en-Y gastric bypass (RYGB).

### Research frontiers

This study aimed to compare the "real-life" weight loss and surgical outcomes of RYGB and LAGB from two large referral bariatric centres in South Australia, in which the databases were prospectively maintained over 10 years.

### Innovations and breakthroughs

In contrast to the impressive weight loss reported by a single Australian LAGB center, the results of the current study are consistent with the available literature on the outcomes of bariatric surgery. The main findings are: (1) RYGB produces substantially greater weight loss and resolution of co-morbidities than LAGB in a community setting, in both the short- and long-term; (2) although peri-operative complications are higher with RYGB than LAGB, which are non-fatal and mostly related to wound infection, the long-term complication rate is higher after LAGB; (3) fasting glucose, total cholesterol and low density lipoprotein levels were significantly lower 12 mo after RYGB than LAGB; and (4) where LAGB fails to induce or maintain weight loss, RYGB appears to be the superior salvage procedure.

### Applications

This study suggests that, in a community setting, RYGB is safe and produces superior short- and long-term weight loss outcomes than LAGB. These data need to be acknowledged and disseminated in the Australian community to allow both surgeons and patients to make an appropriate decision on the choice of bariatric procedure.

### Terminology

LAGB is a weight loss surgical procedure which involves placement of an inflatable band at the top of the stomach to restrict over-eating. In contrast, RYGB involves surgical reconstruction of both the stomach and the small intestine into a small stomach pouch and a bypass of food through the small intestinal. RYGB not only restricts eating but also leads to malabsorption of food.

### Peer review

This is one of the largest trials that compared the "real-life" weight loss and surgical outcomes of the two most commonly performed bariatric procedures in the world, RYGB and LAGB. The results are interesting and suggest that RYGB is safe in a tertiary centre and is superior to LAGB in term of both short- and long-term weight loss outcomes. The data, therefore, enhance the current knowledge in the area of bariatric surgery and allow appropriate decision making in the management of the epidemic obesity.

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## *In vitro* effect of amoxicillin and clarithromycin on the 3' region of *cagA* gene in *Helicobacter pylori* isolates

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### Abstract

**AIM:** To evaluate the *in vitro* effect of amoxicillin and clarithromycin on the *cag* pathogenicity island (*cag* PAI).

**METHODS:** One hundred and forty-nine clinical isolates of *Helicobacter pylori* (*H. pylori*) cultured from gastric biopsies from 206 Colombian patients with dyspeptic symptoms from a high-risk area for gastric cancer were included as study material. Antimicrobial susceptibility was determined by the agar dilution method. Resistant isolates at baseline and in amoxicillin and clarithromycin serial dilutions were subjected to genotyping (*cagA*, *vacA* alleles *s* and *m*), Glu-Pro-Ile-

Tyr-Ala (EPIYA) polymerase chain reaction and random amplified polymorphic DNA (RAPD). Images of the RAPD amplicons were analyzed by Gel-Pro Analyzer 4.5 program. Cluster analyses was done using SPSS 15.0 statistical package, where each of the fingerprint bands were denoted as variables. Dendrograms were designed by following Ward's clustering method and the estimation of distances between each pair of *H. pylori* isolates was calculated with the squared Euclidean distance.

**RESULTS:** Resistance rates were 4% for amoxicillin and 2.7% for clarithromycin with 2% double resistances. Genotyping evidenced a high prevalence of the genotype *cagA*-positive/*vacA s1m1*. The 3' region of *cagA* gene was successfully amplified in 92.3% (12/13) of the baseline resistant isolates and in 60% (36/60) of the resistant isolates growing in antibiotic dilutions. Upon observing the distribution of the number of EPIYA repetitions in each dilution with respect to baseline isolates, it was found that in 61.5% (8/13) of the baseline isolates, a change in the number of EPIYA repetitions lowered antibiotic pressure. The gain and loss of EPIYA motifs resulted in a diversity of *H. pylori* subclones after bacterial adjustment to changing conditions product of antibiotic pressure. RAPD PCR evidenced the close clonal relationship between baseline isolates and isolates growing in antibiotic dilutions.

**CONCLUSION:** Antibiotic pressure does not induce loss of the *cag* pathogenicity island, but it can lead - in most cases - to genetic rearrangements within the 3' region *cagA* of the founding bacteria that can affect the level of tyrosine phosphorylation impacting on its cellular effects and lead to divergence of *cagA*-positive subclones.

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**Key words:** *Helicobacter pylori*; Antimicrobial susceptibility; *cag* pathogenicity island; *cagA* 3' region; Random

amplified polymorphic DNA-polymerase chain reaction

**Core tip:** This study evaluated the *in vitro* effect of amoxicillin and clarithromycin on the *cag* pathogenicity island (*cag* PAI). It was found that the effect of antibiotic pressure does not induce loss of *cag* PAI, but it can lead - in most cases - to genetic rearrangements (loss or gain of Glu-Pro-Ile-Tyr-Ala motif) within the 3' region of *cagA* gene of the founding bacteria that can affect the level of tyrosine phosphorylation impacting on its cellular effects and lead to divergence of *cagA*-positive subclones, which as a set could alter the pathogenic process of *Helicobacter pylori* in cases with treatment failure.

Bustamante-Rengifo JA, Matta AJ, Pazos A, Bravo LE. *In vitro* effect of amoxicillin and clarithromycin on the 3' region of *cagA* gene in *Helicobacter pylori* isolates. *World J Gastroenterol* 2013; 19(36): 6044-6054 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6044.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6044>

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a Gram-negative bacteria in spiral form and microaerophilic that infect the gastric mucosa of approximately 50% of the world's population<sup>[1-4]</sup>. It is usually acquired during childhood<sup>[5]</sup> and when not treated it persists for decades causing clinically asymptomatic chronic gastritis<sup>[6-8]</sup>, although histologically apparent. In a subset of infected individuals, the presence of the bacteria is associated to peptic ulcer, adenocarcinoma, and gastric lymphoma<sup>[3,6]</sup>. Clinical results of the infection are related to the host's immunological defense mechanisms, environmental factors like cigarette smoking and excessive salt intake, diet low in antioxidants, phylogeographic origin<sup>[9]</sup>, and the bacteria's virulence capacity<sup>[8]</sup>. *H. pylori* uses many modalities to colonize the gastric epithelium and some of these adaptation strategies contribute to the progression of the disease<sup>[10]</sup>, among them gene products encoded by *cagA* and *vacA*.

The *cagA* gene is a marker of the *cag* pathogenicity island, present in over 50% of the *H. pylori* strains<sup>[7]</sup>, encodes for the CagA protein one of the main determinants of pathogenicity associated to infection by *H. pylori*<sup>[2,11,12]</sup>, followed by *H. pylori* adhesion to the gastric epithelium, CagA is translocated within the cytoplasm of the epithelial cell via the type IV secretion system, where kinases from the Src and Ab1 family phosphorylate it into specific tyrosine residues inside the Glu-Pro-Ile-Tyr-Ala (EPIYA) repetition motifs<sup>[1,2]</sup>. These EPIYA motifs can be repeated within the protein's variable region<sup>[1]</sup>, and are defined as EPIYA-A, -B, -C, and -D according to the amino acids surrounding them<sup>[2]</sup>. Species of nearby CagA proteins almost always contain EPIYA-A and EPIYA-B sites, followed by one to three repetitions of EPIYA-C in the *H. pylori* Western-type isolates (ABC, ABCC, and ABCCC) or one EPIYA-D site in East Asian-type iso-

lates (ABD). CagA variability with respect to the EPIYA motifs can play an important role in *H. pylori* pathogenesis<sup>[2]</sup>. CagA-positive clinical isolates with increased number of EPIYA motifs obtained from Eastern populations have been associated to higher severity of active chronic gastritis and atrophy. Likewise, an increased number of EPIYA motifs within the Western-type CagA protein has been related to increased levels of phosphorylation, high secretion of interleukin-8, cell elongation, and formation of "hummingbird" phenotype<sup>[1,11]</sup>. Hence, determination of the number of EPIYA motifs within the *cagA* variable region in clinical *H. pylori* isolates is more important than the mere detection of the *cagA*<sup>[1]</sup> and can be useful in predicting the bacteria's pathogenic activity<sup>[11]</sup>.

The *vacA* gene is present in all *H. pylori* strains, although it is only expressed in 50%-65% of them<sup>[5,12]</sup>, it encodes a vacuolating toxin of 88-kDa (VacA) that affects the gastric epithelial cells and is important in the pathogenesis of peptic ulcers and gastric adenocarcinoma<sup>[7]</sup>. Two regions of marked sequence diversity are distinguishable within the *vacA* gene. The *s* region (encodes the signal peptide) is present as *s1* or *s2* allele, while the *m* region (mid region) can be *m1* or *m2*. The combination of the allele mosaic from the *s* and *m* region determines the production of vacuolating cytotoxin and is associated to the bacteria's pathogenicity. The strains harbored *vacA s1m1* have been strongly associated to increased virulence and greater epithelial gastric damage and ulceration than *s2m2* strains<sup>[7,12]</sup>. Thus, *cagA*-positive and *vacA s1m1* genotypes are associated to high risk of gastric cancer<sup>[7]</sup>.

*H. pylori* has extraordinary allelic diversity and genetic variability<sup>[7,13]</sup>, generated through an elevated rate of point mutations, intragenomic and intergenomic recombination<sup>[13]</sup>, phenomena facilitated by the presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced from inflammatory lesions induced by the *H. pylori* infection. This complex environment propitiates a second-order selection that implies variation in mutator genes, creating a non-linear diversification system<sup>[14]</sup>. The fact that virulence factors in *H. pylori* are linked to disease implies that they are a fixed characteristic, but this is not the case and genotype variations can occur through genetic rearrangements<sup>[14]</sup> that eliminates particular immune-stimulating genetic regions (*cag* PAI) or causes variations in potential immune-stimulating molecules (number of EPIYA repetitions in CagA)<sup>[5,13]</sup>, probably reflecting the local selection of *H. pylori* particular phenotypes<sup>[14]</sup> as an escape recourse to the immune response of the host induced by environmental pressures.

This variety of strategies and gene products permit *H. pylori* to persistently colonize its host and cause disease. Within this context, its eradication can contribute to the treatment and prevention of gastric pathologies associated to the infection. Substantial improvements have been accomplished in the efficacy of treatment regimes; nevertheless, all still present faults to completely eradicate the infection<sup>[15]</sup>. This study evaluated the *in vitro* effect of the antibiotics used in standard triple therapy over

specific virulence factors like *cag* PAI, in *H. pylori* isolates from Colombian patients from a high risk region for gastric cancer, to understand the course of the infection in unsuccessful treatments.

## MATERIALS AND METHODS

### Subjects

During 2009, 206 volunteer subjects were recruited with symptoms of dyspepsia, 44.2% (91/206) men ranging in age from 18-68 years, in a population from Túquerres in the high Andes mountains of Colombia, characterized by a high prevalence of *H. pylori* and preneoplastic lesions<sup>[8,16]</sup>. Exclusion criteria included prior gastrectomy, chronic disease, intake of H<sub>2</sub>-receptor antagonists, proton pump inhibitors, or antibiotic intake during the last four weeks prior to endoscopy. During upper gastrointestinal tract endoscopy, biopsies were obtained of gastric antral and body and embedded in paraffin for histopathological evaluation. Additional biopsies, two antral (greater and lesser curvature) and one gastric body (greater curvature) were taken for *H. pylori* culture and immediately frozen in thioglycolate with glycerol. The samples were delivered in liquid nitrogen to the Department of Pathology at Universidad del Valle (Cali, Colombia) for analysis. This research was approved by the Ethics Committee at Universidad del Valle. All the participants provided informed consent.

### Histopathology

Histopathological diagnosis for each participant was independently evaluated by expert pathologists in antral and body gastric sections stained with hematoxylin and eosin, according to the Sydney classification system<sup>[17]</sup>. The categories were non-atrophic gastritis (NAG), multifocal atrophic gastritis without intestinal metaplasia (MAG), intestinal metaplasia (IM), and dysplasia. Cases with discordant diagnosis were again revised and consensus was reached.

### Culture and phenotypic and microscopic identification of *H. pylori*

Fragments of antral and body gastric mucosa were homogenized under sterile conditions in 200 µL of saline solution 0.89% using a homogenizer (Kimble-Kontes, Vineland, NJ, United States). The homogenized was placed on Columbia agar plates (Oxoid, Basingstoke, Hampshire, England) with defibrinated sheep blood at 7% plus selective supplement for *H. pylori* (Dent) containing Vancomycin (10 mg/L), Sodium cefsulodin (5 mg/L), Trimethoprim lactate (5 mg/L), and Amphotericin B (5 mg/L) (Oxoid, Basingstoke, Hampshire, England). The agar plates were incubated under microaerophilic conditions (6% O<sub>2</sub>, 6% CO<sub>2</sub>, 88% N<sub>2</sub>, using CampyPak Plus envelope, BBL, Nashville, TN, United States) at 37 °C from four to eight days until observing small gray translucent colonies. The typical colonies were sub-cultured and later identified as *H. pylori* through their morphology, Gram-

stain, and biochemical analyses for oxidase, catalase, and urease activity.

### Antimicrobial susceptibility in *H. pylori* isolates

Antimicrobial resistance was evaluated by agar dilution method according to guidelines from the Clinical and Laboratory Standards Institute<sup>[18]</sup> using Mueller-Hinton (MHA) agar (Merck KGaA, Darmstadt, Germany) supplemented with defibrinated sheep blood at 7%. Double serial clarithromycin and amoxicillin dilutions (Genfar laboratories, Bogotá, Cundinamarca, Colombia) of 0.25, 0.5, 1.0, 2.0, and 4.0 µg/mL were added to MHA plates. Bacterial isolates sub-cultured in Columbia agar for no more than 72 h were re-suspended in saline solution and turbidity was adjusted at a concentration equivalent to a McFarland 2 standard (containing  $1 \times 10^7$  or  $1 \times 10^8$  CFU/mL), 1 µL of the adjusted inoculum was placed directly onto each MHA agar plate with dilutions of the antibiotic. The minimum inhibitory concentration (MIC) of clarithromycin and amoxicillin was determined after 72 h of incubating the isolates under microaerophilic conditions at 37 °C, and it was recorded as the lowest concentration of the antibiotic that inhibits visible growth of the colonies<sup>[12]</sup>. An isolate was considered resistant to clarithromycin or amoxicillin when its MIC was  $\geq 1.0$  µg/mL. The *H. pylori* reference strain ATTC<sup>®</sup> 43504 was used as quality control strain to monitor MIC precision when using the agar dilution method, considering MIC cutoff points  $< 0.015$  µg/mL as sensitive and  $> 0.12$  µg/mL as resistant to clarithromycin and amoxicillin.

### DNA extraction from *H. pylori* isolates

Bacterial DNA was extracted from pure *H. pylori* cultures that showed resistance to clarithromycin and/or amoxicillin in each of the serial dilutions and from the isolates themselves in baseline (without antibiotic pressure). In all cases, single colonies were taken with swabs and washed in a 1.5 mL tube with 200 µL of sterile saline solution (0.89%). Tube contents were homogenized in vortex for 10 s and centrifuged in a 320R universal micro-centrifuge (Hettich Inc, Tuttlingen, Germany) at 13000 rpm for 2 min at 4 °C. Thereafter, the supernatant was discarded and the bacterial pellet was re-suspended in 300 µL of Lysis buffer (3 µL of Tris HCl 1 mol/L, pH 8.0 (Promega, Madison, WI, United States), 3 µL of EDTA 0.5 mol/L (Calbiochem, Gibbstown, NJ, United States), 15 µL of SDS 10% (Calbiochem, Gibbstown, NJ, United States), 3 µL of proteinase K (10 mg/mL) (Invitrogen, Carlsbad, CA, United States), and 276 µL of distilled water) and incubated in dry well (Labnet, Edison, NJ, United States) at 56 °C for 18 h. Then, the proteinase K was inactivated by heating at 70 °C for 10 min and 120 µL of NaCl 5 mol/L (Calbiochem, Gibbstown, NJ, United States) was added, homogenized by vortex and centrifuged for 5 min at 13000 rpm; the supernatant was transferred to another tube in which two volumes of absolute alcohol were added (Mallinckrodt, St.Louis, Mo, United States), again centrifuged at 13000 rpm for

20 min, then discarding the supernatant and adding 200  $\mu$ L of ethanol at 70%. Once more, it was centrifuged for 5 min at 10000 rpm and then the supernatant was discarded; each tube was dried by inversion at room temperature. Finally, 50  $\mu$ L of TE buffer were added (Tris-HCl 10 mmol/L, pH 8.0 (Promega, Madison, WI, United States), EDTA 1 mmol/L, pH 8.0 (Calbiochem, Gibbstown, NJ, United States) and the DNA was stored at -20 °C. The yield and purity of the DNA was determined by optical density at 260/280 nm in Gene Quant II<sup>®</sup> spectrophotometer (Pharmacia Biotech, Piscataway, NJ, United States) according to manufacturer's instructions.

### Virulence markers for *H. pylori*

To genotype the virulence of *H. pylori* isolates resistant in baseline to clarithromycin and amoxicillin, separate PCR reactions were carried out in a thermocycler (Swift Mini-Pro<sup>™</sup>, Esco Technologies, Hatboro, PA, United States) for *vacA-s* and *vacA-m*, *cagA*, *cag* empty-site. Four sets of primers were initially used in this study. To amplify the *s* region of the *vacA* gene, VA1F and VA1R primers were used, which amplify a fragment of 259 bp (allele *s1*) and 286 bp (allele *s2*). To detect the *m* region of the *vacA* gene, HPMGF and HPMGR primers were employed, which resulted in the amplification of a fragment of 401 bp (allele *m1*) and 476 bp (allele *m2*). The *cagA* gene was amplified to validate the ability of the EPIYA polymerase chain reaction (PCR) to detect the presence of the *cag* PAI, using *cagAF* and *cagAR* primers that amplify a fragment of 183 bp from the 5' end of the *cagA* gene<sup>[19]</sup>. The negative isolates for the *cagA* gene were in all cases confirmed by *cag* empty-site PCR employing ES-F and Rnew-1R primers<sup>[7]</sup>, upon detecting a fragment of 106 bp that indicated the loss of the *cag* PAI. Each experiment included a positive and negative reaction control.

### Amplification of the 3' *cagA* variable region

To detect the presence of the *cag* PAI and characterize the number of EPIYA motifs present in the 3' *cagA* variable region in resistant isolates at each of the antibiotic dilutions (with antibiotic pressure) and in same isolates in baseline (without antibiotic pressure), a PCR reaction was carried out using *cagA2530S* and *cagA3000AS* primers<sup>[11]</sup>. The size of the amplicons expected varied in the range of 370 bp (2 EPIYA motifs), 470 bp (3 EPIYA motifs), 570 bp (4 EPIYA motifs), 670 bp (5 EPIYA motifs)  $\pm$  25 bp and were approximately 100 bp equidistant, indicating the presence of multiple repeated sequences. In cases where a clear band was not evidenced or the presence of amplified, a second PCR was carried out under the same conditions, employing 1  $\mu$ L of the initial amplified, along with the respective controls to detect possible cross contamination. During the development of the trials, *H. pylori* strain 26695 (ATCC 700392) were used (number of access AE000511.1) as size control of the nucleotide sequence encoding for three EPIYA motifs-ABC (470 bp  $\pm$  25 bp), and *H. pylori* strain ATCC 43504 that presents

an EPIYA-ABCCC motif (670  $\pm$  25 bp); thus, predicting the number of repetitions of the EPIYA motifs was possible by direct comparison of the sizes corresponding to PCR amplified.

### RAPD PCR

The genomic differences between *H. pylori* isolates found before and after antibiotic pressure were evaluated by using random primers: 1254 (5'-CCGCAGCCAA-3') and 1281 (5'-AACGCGCAAC-3')<sup>[20]</sup>. These oligonucleotides were amplified in a final 12.5  $\mu$ L reaction volume composed of 2.5  $\mu$ L of PCR buffer (10 mmol/L of Tris-HCl pH 8.0 and 50 mmol/L of KCl-Promega, Madison, WI, United States), 3 mmol/L MgCl<sub>2</sub>, 1 U of Go *taq* polymerase (Promega, Madison, WI, United States), 250  $\mu$ mol/L of dNTPs (Promega, Madison, WI, United States), 25 pmol of each primer, and 1  $\mu$ L of bacterial DNA. The amplification was carried out in a thermocycler (Swift MiniPro<sup>™</sup>, Esco Technologies, Hatboro, PA, United States), prior denaturalization for 5 min at 94 °C, followed by 45 cycles of: 94 °C for 1 min, 36 °C for 1.30 min, 72 °C for 2 min, followed by a final incubation at 72 °C during 10 min. Band size was estimated by using a 100 bp DNA Ladder (Fermentas International Inc., Vilnius, Lithuania). Each isolate was tested with the primers described under the same conditions at least twice and only evident and reproducible bands were evaluated.

### Electrophoresis of amplicons

All the amplicons obtained in each of the previously described PCR reactions were run on agarose gel (SeaKim, FMC Bioloabs) at 2%, stained with ethidium bromide (Invitrogen, Carlsbad, CA, United States) at 0.5  $\mu$ g/mL, in an electrophoresis chamber (Fotodyne Inc., Hartland, WI, United States) at 75 V for 40 min provided by a EC-105 Compact Power Supply (Thermo Fisher Scientific Inc, Asheville, NC, United States).

### Statistical analysis

Initially, univariate and bivariate analyses were conducted; the categorical variables were described as proportions. To determine the statistical significance of the differences in the proportions of *cag* PAI detection *via* *cagA* PCR and EPIYA PCR among baseline resistant isolates and in amoxicillin and clarithromycin dilutions, the McNemar test was employed for data related to the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, United States). Images of the amplicons obtained *via* RAPD PCR were analyzed through the Gel-Pro Analyzer 4.5 program for Windows (Media Cybernetics, Inc, Rockville, MD, United States). Molecular weights of the well-defined, single-pattern bands were analyzed with this program and a matrix of binary data was constructed based on the presence (1) and absence (0) of the bands observed (polymorphic) among the isolates. Ratios among isolates were established through cluster analysis carried out with the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, United States), where each of the fingerprint bands were

**Table 1** Minimum inhibitory concentration of the antibiotics and percentages of resistance among strains

Antibiotic	MIC ( $\mu\text{g/mL}$ )			Resistance ( $n = 149$ )
	MIC <sub>50</sub>	MIC <sub>90</sub>	Range	$n$ (%)
Amoxicillin	2	4	0.25-4.0	6 (4)
Clarithromycin	2	4	0.25-4.0	4 (2.7)

2% (3/149) of the isolates showed double resistance. MIC: Minimum inhibitory concentration.

denoted as variables. The dendrograms were designed by following Ward's clustering method and the estimation of distances between each pair of *H. pylori* isolates was calculated with the squared Euclidean distance. In the clusters, fingerprints with distances less than or equal to five were considered related and distances above five were unrelated. The cluster analysis and the association to antimicrobial susceptibility were evaluated by using the  $\chi^2$  exact. The statistical significance was accepted with a  $P$  value  $\leq 0.05$ .

## RESULTS

Global prevalence of infection by *H. pylori* was 85.4% (176/206) and 72.3% (149/206) by histology and culture, respectively. Isolates positive for *H. pylori* were characterized by antimicrobial susceptibility.

### Histopathology

Among the 176 participants diagnosed through histopathology, 140 (79.5%) presented non-atrophic chronic gastritis, four (2.3%) multifocal atrophic gastritis without intestinal metaplasia, and 32 (18.2%) were diagnosed with multifocal atrophic gastritis with intestinal metaplasia (data not shown).

### Antimicrobial susceptibility

The antimicrobial susceptibility of the 149 isolates is shown in Table 1; MIC<sub>50</sub> and MIC<sub>90</sub> values are indicated. A total of 136 (91.3%) of the bacterial isolates were sensitive to clarithromycin and amoxicillin, while six (4%) of the isolates were AmxR, and four (2.7%) were ClaR. Only three (2%) of the isolates were resistant to both antibiotics. Global resistance was at 8.7% (13/149). The histopathological diagnosis of the participants with isolates that showed resistance was as follows: nine (69.2%) presented non-atrophic chronic gastritis, two (15.4%) had multifocal atrophic gastritis without metaplasia and two (15.4%) had multifocal atrophic gastritis with intestinal metaplasia. It is interesting to find that 50% (2/4) participants diagnosed through histopathology with multifocal atrophic gastritis presented AmxR/ClaR double-resistant isolates (SV323 and SV399) (Table 2).

### Genotyping of virulence genes

The genotyping results of *H. pylori* isolates resistant to amoxicillin and clarithromycin are shown in Table 2. In

23% (3/13) of the participants with isolates resistant to the antibiotics evaluated, colonization was documented with multiple *H. pylori* strains; in one of the cases (SV438) it was impossible to determine the infecting bacterial genotype. It was observed that in all isolates the *cagA*-positive genotype predominated. *vacA s1m1* alleles were predominant, present in 84.6% (11/13), while *s2m2* alleles were only observed in 7.7% (1/13) of the isolates. None of the resistant isolates obtained in this study presented genotypes *s1m2* or *s2m1*.

### Detection of the 3' variable region of the *cagA* gene

Successful amplification took place of the 3' variable region of the *cagA* encoding for EPIYA phosphorylation motifs of the CagA protein in 92.3% (12/13) of the baseline resistant isolates (without antibiotic pressure) and in 60% (36/60) of the resistant isolates growing at each of the serial amoxicillin and clarithromycin dilutions (with antibiotic pressure) (Table 2). This permitted confirming the presence of the *cag* PAI previously detected by *cagA*-specific PCR, as well as characterizing the number of EPIYA motifs present. Amplicon size was in the range of 470-670 bp ( $\pm 25$  bp), according to that suggested by Panayotopoulou *et al.*<sup>11</sup> except for some cases where strong bands were obtained -reproducible with unexpected molecular weights close to 170 and 270 bp ( $\pm 25$  bp) (Figure 1A and Table 2). A single PCR product was observed in 49.3% (36/73) of the isolates obtained between baseline resistant samples and antibiotic dilutions and more than one PCR product with sizes corresponding to different numbers of EPIYA repetitions in 16.43% (12/73) of the isolates. All isolates negative for EPIYA PCR ( $n = 25$ ) were confirmed by *cag* empty-site PCR, finding only four isolates as true *cagA*-negative. When comparing amplification proportions of EPIYA PCR *vs cagA* PCR in detecting *cag* PAI in isolates growing in the amoxicillin and clarithromycin serial dilutions, significant differences were found (McNemar test  $P = 0.006$  and  $P = 0.031$ , respectively), contrasting with the baseline resistant isolates where no significant differences were found between both PCR methods ( $P > 0.05$ ) (Table 3).

### Analysis of RAPD profiles

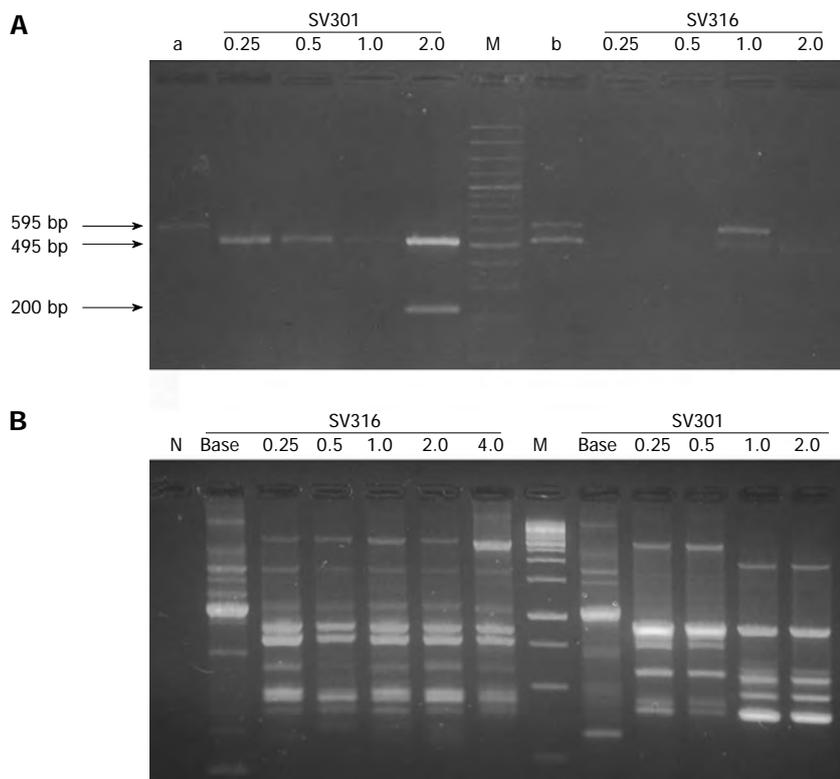
Primers 1281 and 1254 generated a reproducible RAPD fingerprints; they were capable of discriminating 73 different profiles. The number and size of the bands obtained with primer 1281 varied from 3 to 20 and from 76 to 1111 bp, respectively. It was determined if antibiotic pressure was associated to RAPD clusters. Most of the baseline resistant isolates (12/13; 92.3%) and under antibiotic pressure of amoxicillin (18/32; 56.3%) and clarithromycin (18/28; 64.3%) were included in cluster I in comparison to the distribution of these isolates in clusters II and III ( $P = 0.003$ ).

With primer 1254, well-defined patterns were generated of two to 19 fragments in a range from 49 to 1950 bp (Figure 1B). The dendrogram based on RAPD profiles obtained with this primer includes the antimicrobial susceptibility

**Table 2** Distribution of *cagA* genotypes (Glu-Pro-Ile-Tyr-Ala motifs) and *vacA* *s* and *m* among *Helicobacter pylori* isolates obtained from patients with chronic gastritis coming from a region of high risk for gastric cancer, according to profile of *in vitro* antibiotic resistance

Antibiotic resistance	Isolates	Patients (n = 13)	Genotype	Diagnosis	MIC	EPIYA motifs (molecular weight in pairs of bases)					
						Baseline	0.25	0.5	1	2	4
Amoxicillin	Single	SV301	<i>cagA+vacA s1m1</i>	NAG	4	495	495	495	495	495/200	-
		SV310	<i>cagA+vacA s1m1</i>	NAG	2	495	495	495	495	-	-
		SV333	<i>cagA+vacA s1m1</i>	NAG	2	495	495	595/495	595/495	-	-
		SV328	<i>cagA+vacA s1m1</i>	IM	2	695/595/495	495/300/200	495	NA	-	-
		SV338	<i>cagA+vacA s1m1</i>	NAG	2	595/495	NA <sup>2</sup>	NA	NA	-	-
	Mixed	SV316	<i>cagA+vacA s1m1</i>	NAG	> 4	495	NA	NA	595/495	495	NA
Clarithromycin	Single	SV336	<i>cagA+vacA s1m1</i>	NAG	2	595	595	595	595	-	-
		SV440	<i>cagA+vacA s2m2</i>	NAG	> 4	595	595/495	595/495	NA	NA	NA
	Mixed	SV415	<i>cagA+vacA s1m1</i>	NAG	> 4	495	495	495	495	495	2NA
	SV438	Undefined	IM	2	NA <sup>2</sup>	NA <sup>1</sup>	NA <sup>1</sup>	NA	-	-	
	SV323	<i>cagA+vacA s1m1</i>	MAG	2	495	NA	NA	495/200	-	-	
Amoxicillin + Clarithromycin	Single	SV399	<i>cagA+vacA s1m1</i>	MAG	2	495	NA	495	495	-	-
					2		495/200	NA	495	-	-
		SV433	<i>cagA+vacA s1m1</i>	NAG	> 4	495	NA	1NA	495	595/495	200
				> 4		495	495	495	495	495	

<sup>1</sup>Positive for empty-site PCR; <sup>2</sup>Negative for EPIYA PCR, *cagA*-specific PCR and *cag* empty-site PCR. NA: Did not amplify; PCR: Polymerase chain reaction; EPIYA: Glu-Pro-Ile-Tyr-Ala; NAG: non-atrophic gastritis; MIC: Minimum inhibitory concentration; IM: Intestinal metaplasia; MAG: Multifocal atrophic gastritis without intestinal metaplasia.



**Figure 1** Electrophoretic analysis. A: EPIYA polymerase chain reaction (PCR) products from DNA of the *Helicobacter pylori* (*H. pylori*) isolates SV301 and SV316 that grew in the Amoxicillin serial dilutions, the PCR products were analyzed in agarose gel at 2%. Lines a and b positive controls; line a: Clinical *H. pylori* isolate (PZ5085) with EPIYA ABCC motif (570 ± 25 bp) confirmed by sequencing; Line b: A mix of DNA from isolate PZ5085 and from the *H. pylori* strain 26695 with EPIYA-ABC motif (470 ± 25 bp). M: 100 bp DNA ladder. Distribution of different molecular weights among isolates is an indication of the presence of multiple EPIYA repetitions; B: Random amplified polymorphic DNA fingerprints generated with primer 1254 in *H. pylori* isolates resistant in baseline (without antibiotic pressure) and resistant isolates growing in each of the serial antibiotic dilutions (with antibiotic pressure); line N: negative reaction control; M: 100 bp ladder.

for each isolate (Figure 2). Cluster analyses for this primer showed three main clusters in a parsimonious solution. In

none of the clusters was the segregation of the baseline isolates and under antibiotic pressure significant ( $P = 0.704$ ).

**Table 3** Detection of *cag* pathogenicity island in *Helicobacter pylori* isolates resistant in baseline and in antibiotic dilutions by amplification of the *cagA* gene *n* (%)

Method	Detection of the <i>cag</i> PAI		
	Baseline ( <i>n</i> = 13)	Dilutions	
		Amoxicillin ( <i>n</i> = 32)	Clarithromycin ( <i>n</i> = 28)
<i>cagA</i> PCR	11 (84.6)	30 (93.8)	22 (78.6)
EPIYA PCR	12 (92.3)	20 (62.5)	16 (57.1)
<i>P</i> value	> 0.05	0.006	0.031

PAI: Pathogenicity island; PCR: Polymerase chain reaction; EPIYA: Glu-Pro-Ile-Tyr-Ala.

### Effect of antibiotic pressure on the number of EPIYA repetitions

Upon observing the distribution of the number of EPIYA repetitions in each of the serial dilutions of the antibiotics with respect to the baseline isolates, change at the number of EPIYA repetitions was found in 61.5% (8/13) of the baseline isolates under antibiotic pressure. In six of these isolates (SV301, SV328, SV440, SV323, SV399 y SV433), the change consisted in loss of EPIYA repetitions evidenced by the low molecular weight of the amplified bands. In contrast, two isolates (SV316 and SV333) revealed gain of EPIYA repetitions. The gain and loss of EPIYA motifs resulted in a diversity of *H. pylori* subclones after bacterial adjustment to changing conditions product of antibiotic pressure. In all cases, the genotyping results and the presence of a single band in the EPIYA PCR for the baseline isolate permitted discarding the presence of more than one infecting *cagA*-positive isolate, except for isolate SV328 that presented in baseline three CagA species that differed in the number of EPIYA repetitions; after antibiotic pressure, alteration of the number of EPIYA repetitions was evidenced in two of the CagA species present (MIC 0.25 µg/mL) and its subsequent loss with increased concentration of the antimicrobial (MIC 0.5 µg/mL). Also, in three isolates no change was noted in the 3' variable region of the *cagA* and in the two remaining cases (SV338 and SV438) change could not be documented due to absence of EPIYA PCR amplification. We used RAPD PCR to evaluate the clonal relationship of the isolates and verify that these strains had the same origin. Specifically, the RAPD fingerprints generated by both primers reflected a close clonal relationship between baseline isolates (without pressure) and isolates growing in antibiotic dilutions (under pressure).

## DISCUSSION

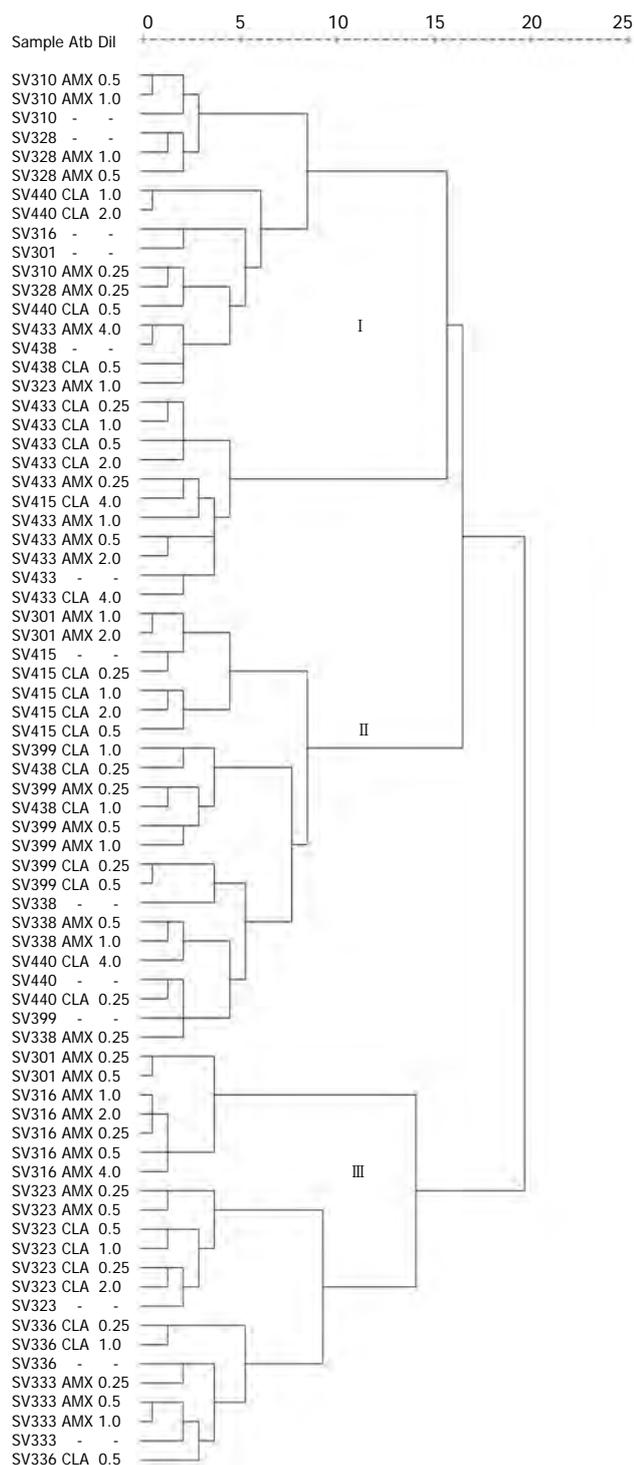
Gastric infection by *H. pylori* is a prominent risk factor for gastric cancer; the outcome of the infection is determined by the pathogen's characteristics in combination with environmental and host factors. It has been demonstrated that the precursor lesions that lead to the development of intestinal-type gastric adenocarcinoma have a series of sequential changes in the gastric mucosa<sup>[21]</sup>. The

prolonged process of these precancerous lesions provides an opportunity to prevent the progression to more advanced stages; hence, efforts must be aimed at primary prevention. Anti-*H. pylori* treatment regimes combine two or more antibiotics with a proton pump inhibitor; however, they are not 100% effective in infection resolution<sup>[12]</sup>. The purpose of this research was to evaluate the *in vitro* effect of amoxicillin and clarithromycin on *cag* PAI and the 3' variable region of the *cagA* of *H. pylori*, to understand the course of the infection in unsuccessful treatments.

We found *H. pylori* prevalence of 85.4% and 72.3% through histology and culture, respectively. Differences between the results could be due to the unequal distribution of *H. pylori* in the stomach. These results also coincide with the prevalence of the infection (73.8%) previously reported by Bravo *et al.*<sup>[16]</sup> using histology within the same community. Differences observed through histology between both studies can be due to participant age and sex. Some 79.5% of the subjects diagnosed via histopathology presented non-atrophic chronic gastritis and the proportion observed of gastric cancer precursor lesions was low; findings unexpected in a population at high risk for gastric cancer. This could be due to sample bias; over half (55.8%) of the subjects recruited were women and the intestinal-type gastric adenocarcinoma is predominant in men<sup>[5]</sup>.

Also, a low resistance rate to clarithromycin was noted in this population (2.7%), which agrees with the resistance rate reported in another Colombian region with similar geographic conditions, Pereira (2.2%)<sup>[22]</sup>, and other regions in Latin America<sup>[23,24]</sup>, but differing from the rates reported in Ecuador (9.5%)<sup>[25]</sup>, Brazil (9.8%), the metropolitan region in Chile (20%)<sup>[26]</sup>, Mexico (25%)<sup>[27]</sup>, and Argentina (27.7%)<sup>[12]</sup>. A relatively high resistance rate was also noted for amoxicillin (4%), similar to rates documented within the country by other authors in patients with dyspepsia symptoms in Bogotá (3.8%)<sup>[28]</sup> and (7%)<sup>[29]</sup>, although contrasting with the results obtained by Álvarez *et al.*<sup>[22]</sup> who found no isolates resistant to amoxicillin in Pereira, agreeing with that described in other parts of the world, where resistance rates to this antibiotic are null or below 2%<sup>[12,23,30,31]</sup>, implying an added difficulty to manage infection by *H. pylori* in our environment. In general, increased resistance to these two antibiotics could be a risk factor to consider for unsuccessful treatment within this population; thus, inasmuch as treatment is based on tests to antibiotic susceptibility, it may be more efficient in achieving high eradication rates. Additionally, studies conducted by our group have found that cases where treatment was not successful was associated to increased prevalence of less virulent strains<sup>[15,32]</sup>; it is not clearly known if this phenomenon is because of the effect of positive selection of certain genotypes (*cagA*-negative/*vacA* *s2m2*) in cases of multiple infection or because of loss of specific virulence factors (*cag* PAI) induced by antibiotic pressure, and this is the question we seek to respond.

The combination of different *cagA* genotypes and



**Figure 2** Dendrogram of the profile of random amplified polymorphic DNAs generated with primer 1254 in *Helicobacter pylori* isolates resistant in baseline and resistant isolates growing at each of the serial dilutions of amoxicillin and clarithromycin. Three separate clusters (I, II and III) are indicated. In none of the clusters was segregation of sensitive and resistant isolates significant ( $P = 0.704$ ). Cluster analyses were designed by following Ward's hierarchical clustering method and estimation of distances between each pair of *Helicobacter pylori* (*H. pylori*) isolates were calculated with the squared Euclidean distance. Distances between isolates are given in a 25-point standardized scale that reflects with sufficient precision the existing proportionality between the original distances. The fusions produced near the origin of the scale (left) indicate that the cluster formed is quite homogeneous. Conversely, fusions produced on the final zone of the scale (right) indicate the cluster formed is quite heterogeneous. Atb: Antibiotic, Dil: Dilution.

*vacA s* and *m* alleles illustrate the genomic variability of *H. pylori*<sup>[12]</sup>. This study found high prevalence of the *cagA* gene and *vacA s1m1* alleles in baseline amoxicillin and clarithromycin resistant isolates; these observations agree with previous findings reported by Sicinski *et al*<sup>[7]</sup> who genotyped gastric biopsies embedded in paraffin of two Colombian populations with contrast in the risk of gastric cancer, finding that the most virulent *cagA*-positive/*vacA s1m1* strains are more prevalent in the high-risk area. In 23% of these resistant isolates, genotyping of *cagA* and *vacA* permitted our documenting infection by multiple strains of *H. pylori*. Mixed infection with two or more *H. pylori* strains colonizing the same patient possibly represent a stable association during long-term colonization.

Also, although no theoretical bases exist to predict an association between antibiotic resistance and virulence markers, several studies have been carried out to find an association. Most studies have shown no significant correlation<sup>[33]</sup>; however, studies conducted in northern Wales<sup>[34]</sup>, central Italy<sup>[35]</sup> and western Argentina<sup>[12,36]</sup> found a clear association between virulent genotypes and antibiotic resistance. The correlation between virulence factors and antimicrobial resistance varies in different countries and although we did not evaluate this possible relationship, we found that the *cagA*-positive/*vacA s1m1* genotype predominated in resistant isolates; nevertheless, this could be a product of the high prevalence of circulating virulent strains in this region<sup>[2,7,16]</sup> and not a reflection of a possible association between virulence factors and resistance.

Over 98% of the *cagA*-positive isolates harbored a CagA protein with three (495 bp) or four (595 bp) EPIYA motifs located quite probably on the ABC or ABCC combination, respectively. Although in none of the cases were PCR products sequenced, with this methodology we can certainly predict the presence of these combinations, as demonstrated by that reported by Panayotopoulou *et al*<sup>[11]</sup>. Nonetheless, in amplicons with unexpected sizes of 170 and 270 bp (+25 bp) and over four EPIYA repetitions (695 bp), sequencing is indispensable to establish the exact type of EPIYA motif combinations; although in the last case, predicting the correct number of EPIYA motifs was possible, being one of the limitations in our study. In no case do we expect to find East Asian-type strains with EPIYA-D motif circulating in the population, although the primers used were also designed for its detection, these predictions are supported by a prior study performed by our group with 41 individuals from the same location, which evaluated through sequencing the variations in the *cagA* carboxyl-terminal end, which varied considerably in size, number, and structure of EPIYA motifs (ABC, ABCC, ABCBC, ABCC, or ABCCC) and no *H. pylori* strain contained an EPIYA-D motif<sup>[2]</sup>.

A total of 25 cases were EPIYA PCR negative and were then confirmed by *cagA*-specific PCR and *cag* empty-site PCR, finding only four of them as true *cagA*-negative,

which evidences: (1) the low sensitivity of EPIYA PCR compared to *cagA* PCR, which contrasts with the observations by Panayotopoulou *et al.*<sup>[11]</sup> who suggest that the lack of amplification of the EPIYA PCR can precisely identify the *cagA*-negative cases. Perhaps, the differences observed in our case can be the effect of the random degradation of the bacterial DNA extracted from some samples -hindering amplification of large-size fragments (> 200 bp); in any case, it is wise to validate the detection of the *cagA* gene by *cagA*-specific PCR; and (2) the inefficiency of the DNA extraction method used in those cases where amplification was not obtained<sup>[37]</sup>. However, EPIYA PCR is a simple strategy to detect the *cagA* status and for precise prediction of the number and type of EPIYA motifs, involving a single amplification step<sup>[11]</sup> and permitting rapid screening of multiple isolates. Additionally, the EPIYA PCR facilitated the identification of *cagA*-positive *H. pylori* subclones closely related within the same isolate, harbored different number of EPIYA repetitions, indistinguishable in genotyping.

Because of the methodological approach employed in this study, it was possible to document *in vitro* that the effect of antibiotic pressure does not induce loss of *cag* PAI, but it can lead - in most cases - to genetic rearrangements within the 3' region of *cagA* gene of the founding bacteria that produces a non-directed alteration (loss or gain) of EPIYA motifs. *H. pylori* has shown a unique genetic variability and these genetic changes could hypothetically be guided by intra-specific homologous recombination<sup>[11,14]</sup> and favored by their panmictic population structure<sup>[3,6]</sup>, permitting the bacteria to quickly adapt to changing conditions, being able to: (1) eliminate particular immune-stimulating genetic regions (EPIYA motifs) that would result in the production of a non-phosphorylatable form of CagA that does not induce profound morphological changes in the host cell<sup>[38]</sup> supported by amplicons of unexpected molecular weights (170 and 270 bp) obtained in this study; and (2) induce variations within the 3' terminal of the *cagA* encoding sequence that lead to differentiation in subclones with different numbers of EPIYA repetitions that can act as potential reservoirs of genetic elements for their cohabitants, with dominant clones favored by natural selection and additionally serve as escape recourse to the host's immune response; thus, increasing their possibilities to adapt, evolve, and persist. In cases in which the 3' variable region of the *cagA* remained intact following antibiotic pressure, suggests the presence of unknown extrinsic factors that can be influencing the phenomenon.

In most cases, the RAPD fingerprints confirmed the close clonal relationship existing among the isolates growing under antibiotic pressure and their corresponding baseline isolate, gathering them within the same cluster; for this reason, the cluster analysis for primer 1254 did not show differences in the segregation of isolates without pressure (baseline) and with pressure (AmxR and/or ClaR) among the three clusters formed, and although the cluster analysis based on the profile generated by the

other primer (1281) did reveal significant differences ( $P = 0.003$ ) among the three clusters, the differences observed are attributed to gathering most of the isolates within cluster I, without discriminating between isolates without pressure and those under pressure, likewise, always gathering the isolates under antibiotic pressure along with the baseline isolate. Some isolates under specific antibiotic concentration were not grouped along with their other clones at different antibiotic concentrations within the same cluster, probably due to the presence of more than one subclone within that isolate that induced a distortion in the RAPD pattern with this being a technique that generates a profile based on the complete DNA. Even though these observations agree with that expected, no differences should exist between isolates growing in each of the antibiotic dilutions and the baseline isolate because they are the same isolate.

These findings are specially important because of their possible implications for the treatment and evolution of gastro-duodenal diseases caused by *H. pylori*, suggesting that acquisition or deletion of EPIYA motifs product of the antibiotic effect can affect the phosphorylation level of tyrosine, impacting on its cellular effects<sup>[11]</sup> and leading to the divergence of *cagA*-positive *H. pylori* subclones, which as a set could alter the pathogenic process of *H. pylori* in cases with treatment failure. Such pool of *H. pylori* clones may exist in dynamic equilibrium within any potential host<sup>[11]</sup>, cooperating through quorum sensing and recombination, which can lead to down-regulation of interaction with the host<sup>[14]</sup> to induce lesser damage, even when some damage is inevitable, given that in these subclones the divergent CagA species would be normally expressed by inducing several degrees of "hummingbird" phenotype upon the infected epithelial cells<sup>[11]</sup>. This hypothesis could explain - in part - the observations registered by Mera *et al.*<sup>[39]</sup> who during a follow up of a cohort for 12 years found that those subjects receiving anti-*H. pylori* treatment but remaining infected showed decreased histopathological score -0.19 over the average histopathology score at baseline. Additional *in vitro* studies including a higher number of resistant isolates are necessary to determine the direct effect of clarithromycin and amoxicillin upon the *cagA* gene and evaluate their effect on the *vacA* gene, which was not considered in this study, even though it is a gene susceptible to genetic modifications according to that reported by Argent *et al.*<sup>[40]</sup>. Likewise, new studies are needed aimed at evaluating the *in vivo* effect of anti-*H. pylori* treatment upon virulence factors previously described in *H. pylori* isolates from patients with treatment failure and deciphering the complex molecular mechanisms through which these genomic alterations are produced. Upon proving its effect on *cagA*, this methodological approach could constitute a useful prognosis tool in clinical practice, when detecting the presence of multiple subclones and determining the number and type of EPIYA motifs, permitting the prediction of pathogenic activity of the bacteria in unsuccessful treatments.

Finally, given the generalized use of antibiotics in our environment to combat diverse infectious agents, it is tempting to speculate that inadequate treatments (not *H. pylori*-targeted) can, likewise, significantly impact upon *H. pylori* strains co-existing along with their host.

appropriate, results are clearly presented and conclusions are corroborated by the results.

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## COMMENTS

### Background

*Helicobacter pylori* (*H. pylori*) is a prominent risk factor for gastric cancer, higher risk in individuals infected with *cagA*-positive strains. Its eradication with antibiotics constitutes a chemoprevention strategy; however, treatments are not completely effective and seem to contribute to increasing the prevalence of less virulent strains.

### Research frontiers

Virulence-associate genotypes of *H. pylori* are increasingly recognized as determinants of the clinical outcome of the infection. The fact that virulence factors are linked to disease implies that they are a fixed characteristic, but this is not the case and genotype variations can occur through genetic rearrangements that either eliminates particular immunostimulatory genetic regions (*cag* pathogenicity island) or causes variations in potential immunostimulatory molecules (number of Glu-Pro-Ile-Tyr-Ala motifs in *CagA*) probably reflecting the local selection of *H. pylori* particular phenotypes as an escape resource to the immune response of the host induced by environmental pressures.

### Innovations and breakthroughs

Some studies indicate that failure to eradicate *H. pylori* from the stomach may result in an increase in the prevalence of less virulent genotypes. It is unknown if this phenomenon is due to the positive selection of certain genotypes (*cagA*-negative/*vacA s2m2*) in cases of multiple infection or loss of specific virulence factors (*cag* PAI) induced by antibiotic pressure. This study shows that antibiotic pressure does not induce loss of the *cag* PAI, but it can lead to genetic rearrangements within of the *cagA* variable region that can affect the level of tyrosine phosphorylation impacting on its cellular effects and lead to divergence of *cagA*-positive subclones, which as a set could alter the pathogenic process of *H. pylori* in cases with treatment failure.

### Applications

These *in vitro* findings are specially important because of their possible implications for the treatment and evolution of gastro-duodenal diseases caused by *H. pylori*, suggesting that acquisition or deletion of EPIYA motifs product of the antibiotic effect can affect the phosphorylation level of tyrosine and leading to the divergence of *cagA*-positive *H. pylori* subclones. Further studies are needed aimed at evaluating the effect of anti-*H. pylori* treatment on the *cagA* variable region in *H. pylori* isolates from patients with treatment failure and deciphering the complex molecular mechanisms through which these genomic alterations are produced. Upon proving its effect on *cagA*, this methodological approach could constitute a useful prognosis tool in clinical practice, when detecting the presence of multiple subclones and determining the number and type of EPIYA motifs, permitting the prediction of pathogenic activity of the bacteria in unsuccessful treatments.

### Terminology

The *cag* pathogenicity island is a genomic fragment of approximately 40 kb that contains 30 genes that encode a type IV secretion apparatus, which export proteins from bacterial cytoplasm to host epithelial cells. The terminal gene in the island, *cagA*, is commonly used as a marker for the entire *cag* locus.

### Peer review

This manuscript deals with the evaluation of the *in vitro* effect of amoxicillin and clarithromycin on the *cag* PAI. The authors found that antibiotic pressure does not induce loss of the *cag* PAI and leads in most cases to genetic rearrangements within the 3' region of *cagA* of the bacteria that lead to divergence of *cagA*-positive subclones. This study is interesting and original. Methods are

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## Liver metastasis is the only independent prognostic factor in AFP-producing gastric cancer

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### Abstract

**AIM:** To investigate differences between common gastric cancer and  $\alpha$ -fetoprotein (AFP)-producing gastric cancer according to the presence or absence of liver metastasis.

**METHODS:** Between 1997 and 2011, 1299 patients underwent gastrectomy for gastric cancer (GC) at our institute and their hospital records were reviewed retrospectively. Patients were immunohistochemically divided into two groups: 23 patients (1.8%) with AFP-producing GC and 1276 patients (98.2%) without it.

**RESULTS:** AFP-producing GC patients had a significantly higher incidence of deeper tumors, venous invasion, lymphatic invasion, lymph node metastasis, and liver metastasis and a poorer prognosis ( $P < 0.005$ ) than those without AFP-producing GC. However, multi-

variate analysis revealed that AFP-positivity was not an independent prognostic factor. The prognosis of AFP-producing GC was similar to that of AFP-non producing GC according to the presence or absence of liver metastasis. Concerning recurrence, 47.8% of patients (11/23) with AFP-producing GC and 20.0% of patients (256/1276) without AFP-producing GC exhibited recurrence. Liver metastasis [90.9% (10/11)] was the most prevalent in AFP-producing GC patients. Multivariate analysis revealed that liver metastasis was the only independent prognostic factor in AFP-producing GC (HR = 17.6, 95%CI: 2.1-147.1;  $P = 0.0081$ ).

**CONCLUSION:** AFP-producing GC is similar to common GC without liver metastasis, which should be specifically targeted in an effort to improve the prognosis of AFP-producing GC patients.

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**Key words:**  $\alpha$ -fetoprotein; Gastric cancer; Liver metastasis; Poor prognosis; Immunostaining

**Core tip:** In the present study, we re-evaluated the clinicopathological characteristics and clinical outcomes of consecutive patients with  $\alpha$ -fetoprotein (AFP)-producing gastric cancer (GC). The results obtained clearly demonstrated that clinical behaviors were different between patients with and without AFP-producing GC. However, the prognosis according to the presence or absence of liver metastasis was similar between patients with and without AFP-producing GC. Our results show that liver metastasis should be specifically targeted in an effort to improve the prognosis of AFP-producing GC.

Hirajima S, Komatsu S, Ichikawa D, Kubota T, Okamoto K, Shiozaki A, Fujiwara H, Konishi H, Ikoma H, Otsuji E. Liver metastasis is the only independent prognostic factor in AFP-producing gastric cancer. *World J Gastroenterol* 2013;

## INTRODUCTION

$\alpha$ -fetoprotein (AFP) was initially found in the human fetus in 1956 and is normally produced in the fetal liver and yolk sac<sup>[1]</sup>. AFP is considered to be a useful tumor marker in screening or monitoring patients with hepatocellular carcinomas or yolk sac tumors. Previous studies have shown that AFP may be produced in other cancers including primary gastric cancer (GC)<sup>[2-6]</sup>.

The incidence of AFP-producing GC is low and has been reported to be 1.3%-15% in GC<sup>[7-11]</sup>. Since AFP-producing GC was first described in 1970 by Bourrille *et al.*<sup>[12]</sup>, several cases of early and advanced AFP-producing GC have since been reported. Most of them exhibited a poor prognosis with a high incidence of lymphatic invasion, venous invasion, and synchronous and metachronous liver metastasis. Therefore, AFP-producing GC has been associated with a poorer prognosis than AFP-non producing GC<sup>[12-16]</sup>. However, most of these studies were restricted to the overall prognosis, and few studies conducted subgroup analyses with special reference to the presence or absence of liver metastasis.

In the present study, we re-evaluated the clinicopathological characteristics and clinical outcomes of consecutive patients with AFP-producing GC. The results obtained clearly demonstrated that clinical behaviors were different between patients with and without AFP-producing GC. However, the prognosis according to the presence or absence of liver metastasis was similar between patients with and without AFP-producing GC. It is important to note that the overall poorer prognosis of AFP-producing GC is not related to difficulties in treating it, but from the lack of effective and recommended treatments for liver metastasis of common GC. Our results show that liver metastasis should be specifically targeted in an effort to improve the prognosis of AFP-producing GC.

## MATERIALS AND METHODS

### Patients

Between 1997 and 2011, 1375 patients with histologically confirmed primary gastric adenocarcinoma underwent gastrectomy at the Department of Digestive Surgery, Kyoto Prefectural University of Medicine. A total of 76 GC patients with active or chronic hepatitis, fatty liver, and hepatocellular carcinoma were excluded from this analysis, and 1299 patients were enrolled in this study and their hospital records were reviewed retrospectively.

Resected specimens were examined by pathologists based on classifications of the 14th Japanese Classification of Gastric Carcinomas (JCGC)<sup>[17]</sup> and 7<sup>th</sup> tumor-node-metastasis (TNM)<sup>[18]</sup>. The clinicopathological find-

ings of these patients were determined retrospectively on the basis of their hospital records. Histological types were classified as differentiated (papillary, moderately, and well-differentiated adenocarcinoma) and undifferentiated (poorly, undifferentiated, signet-ring cell carcinoma, and mucinous adenocarcinoma) based on the 14<sup>th</sup> JCGC.

In this study, specimens were fixed in a 10% neutral buffered formaldehyde solution and embedded in paraffin. They were then stained with hematoxylin and eosin and examined histologically. They were also examined immunohistochemically to determine the presence of the tumor-associated antigen AFP. Immunohistochemical analyses were performed using the streptavidin-biotin method. Sections were dewaxed in xylene, passed through ethanol, and incubated with 3% hydrogen peroxide in methanol for 4 min to block endogenous peroxidase activity. Sections were washed in phosphate-buffered saline (PBS) and 10% normal rabbit serum was applied for 20 min to reduce nonspecific antibody binding. AFP, a rabbit polyclonal antibody purchased from the DAKO Corporation, was diluted 1:100 and reacted with tissue specimens at room temperature for 2 h. Specimens were washed three times with PBS. Sections were incubated with biotinylated rabbit antimouse immunoglobulin G at a dilution of 1:100 for 30 min followed by three washes. Slides were reacted with a streptavidin-biotin peroxidase reagent for 30 min at a dilution of 1:100 and washed with PBS three times. Hepatocellular carcinoma cell tissues containing AFP were used as positive controls. The first antibody was replaced with a sodium phosphate buffer for negative controls. Consequently, the immunoreactivity of AFP was demonstrated in 23 primary lesions of GC patients.

The follow-up program schedule for all patients comprised a regular physical examination and laboratory blood tests including tumor markers such as CEA and CA19-9, chest X-rays every 3 mo in the first postoperative year, every 6 mo in the second post-operative year, and annually thereafter for at least 5 years. Computer tomography (CT) was performed annually in patients with pathological stage I - II tumors and every 6 mo in patients with more than pathological stage III tumors for the first 5 years. Otherwise, CT was performed within one month when elevations in tumor markers and/or symptoms were detected in follow-up laboratory blood tests and regular physical examinations. Endoscopy was performed in all patients annually to screen for cancer in the gastric remnant.

### Statistical analysis

The  $\chi^2$  test and Fisher's exact probability test were performed to compare clinicopathological characteristics between each group. Cause-specific death was recorded when the cause of death was specified as recurrent gastric cancer. Post-recurrence survival from recurrence to death, and overall survival from curative gastrectomy to death were estimated using the Kaplan-Meier method, and the log-rank test was used to assess differences be-

**Table 1 Association between clinicopathological characteristics and  $\alpha$ -fetoprotein expression *n* (%)**

Characteristic	<i>n</i>	Gastric cancer		<i>P</i> value <sup>1</sup>
		AFP-producing	AFP-non producing	
Total	1299	23	1276	
Sex				0.9974
Male	875	16 (70)	859 (67)	
Female	424	7 (30)	417 (33)	
Age (yr)				0.5835
Mean	64.5			
	(range: 27-94)			
< 65	610	9 (39)	601 (47)	
≥ 65	689	14 (61)	675 (53)	
Location				0.8753
Upper	292	4 (17)	288 (23)	
Middle	591	10 (44)	581 (45)	
Lower	416	9 (39)	407 (32)	
Macroscopic type				< 0.0001
Type 0-1 / II / III	690	2 (9)	688 (54)	
Type 1/2/3/4	609	21 (91)	588 (46)	
Histopathological grading				0.8155
Differentiated	596	10 (43)	586 (46)	
Undifferentiated	703	13 (57)	690 (54)	
Tumor size (mm)				0.0073
< 40	614	4 (17)	610 (48)	
≥ 40	685	19 (83)	666 (52)	
Venous invasion				< 0.0001
Negative	937	5 (22)	932 (73)	
Positive	362	18 (78)	344 (27)	
Lymphatic invasion				0.0071
Negative	726	6 (26)	720 (56)	
Positive	573	17 (74)	556 (44)	
TNM classification				
pT categories				0.036
T1	664	4 (17)	660 (52)	
T2	122	3 (13)	119 (9)	
T3	217	7 (31)	210 (16)	
T4	296	9 (39)	287 (23)	
pN categories				0.0007
N0	821	9 (39)	812 (63)	
N1	140	9 (39)	131 (10)	
N2	137	3 (13)	134 (11)	
N3	201	2 (9)	199 (16)	
pStage				0.0002
I	769	3 (13)	766 (60)	
II	137	3 (13)	134 (11)	
III	195	9 (39)	186 (14)	
IV	198	8 (35)	190 (15)	
Absent	955	12 (52)	943 (74)	0.0192
Present	344	11 (48)	333 (26)	
Liver metastasis				< 0.0001
Absent	1245	13 (57)	1232 (97)	
Present	54	10 (43)	44 (3)	

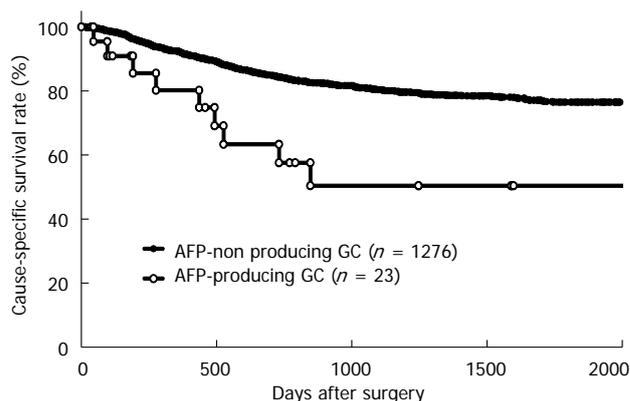
Significantly different values are highlighted using boldface type. <sup>1</sup>*P* values are from  $\chi^2$  or Fisher's exact test and were significant at < 0.05. AFP:  $\alpha$ -fetoprotein; TNM: Tumor, nodes, metastasis.

tween clinical factors. A *P*-value less than 0.05 was considered significant.

## RESULTS

### Clinicopathological characteristics of patients with primary AFP-producing GC in GC

The mean patient age was 64.5 (range 27-94) years, and



**Figure 1** Survival curves between  $\alpha$ -fetoprotein-producing gastric cancer and  $\alpha$ -fetoprotein non producing gastric cancer. The postoperative cause-specific 5-year survival rate of patients with  $\alpha$ -fetoprotein (AFP)-producing gastric cancer (GC) was significantly poorer than that of patients without AFP-producing GC (5-year survival rate: 50.3% and 76.5%, *P* = 0.002).

the male: female ratio was 2.1:1. Clinicopathological characteristics were compared between AFP-producing GC and AFP-non producing GC groups (Table 1). Patients with AFP-producing GC had a significantly higher incidence of venous invasion (*P* < 0.0001), lymphatic invasion (*P* = 0.0071), larger tumor size (*P* = 0.0073), lymph node metastasis (*P* = 0.0007), liver metastasis (*P* < 0.0001), and advanced stage (*P* = 0.0002) than those with AFP-non producing GC. The postoperative cause-specific 5-year survival rate of patients with AFP-producing GC was significantly poorer than that of patients without AFP-producing GC (5-year survival rate: 50.3% and 76.5%, *P* = 0.002) (Figure 1).

### Univariate and multivariate survival analyses in 1299 GC patients

Of all 1299 GC patients analyzed, univariate analysis showed that age, location, macroscopic type, tumor size, histological type, venous invasion, lymphatic invasion, pT categories, pN categories, the presence of AFP-producing GC, and liver metastasis were prognostic factors (Table 2). Furthermore, multivariate analysis using stepwise Cox regression procedures demonstrated that macroscopic type, tumor size, venous invasion, lymphatic invasion, pN categories, and liver metastasis were independent prognostic factors. However, the presence of AFP-producing GC was not an independent prognostic factor (Table 2).

### Comparison of recurrence patterns between patients with and without AFP-producing GC

To develop surveillance programs and treatment strategies, we compared recurrence patterns between patients with and without AFP-producing GC. Distant metastasis and recurrence developed in 47.8% of patients (11/23) with AFP-producing GC and 20.0% of patients (256/1276) without AFP-producing GC. Of these, liver metastasis [90.9% (10/11)] was the most prevalent in AFP-producing GC patients, while peritoneal recurrence

**Table 2** Univariate and multivariate survival analyses in 1299 gastric cancer patients

Factor	Univariate <sup>1</sup>		Multivariate <sup>2</sup>	
	P value	HR	95%CI	P value
Sex				
Male vs female	0.3121			
Age (yr)				
65 ≤ vs < 65	0.0033			
Location				
U vs ML	0.0027			
Macroscopic type				
Type 1/2/3/4	< 0.0001	3.760	2.012-7.013	< 0.0001
vs Type 0- I / II / III				
Tumor size (mm)				
40 ≤ vs < 40	< 0.0001	2.128	1.259-3.597	0.0048
Histological type				
Undifferentiated	0.014			
vs Differentiated				
pT categories				
T2/3/4 vs T1	< 0.0001			
Venous invasion				
Positive vs Negative	< 0.0001	1.721	1.247-2.375	0.0010
Lymphatic invasion				
Positive vs Negative	< 0.0001	2.793	1.656-4.717	0.0001
pN categories				
Positive vs Negative	< 0.0001	2.513	1.614-3.951	< 0.0001
AFP producing vs non producing	0.0018			
Liver metastasis				
Positive vs Negative	< 0.0001	3.152	2.116-4.558	< 0.0001

<sup>1</sup>The Kaplan-Meier method, and significance was determined by the log-rank test; <sup>2</sup>Multivariate survival analysis was performed using Cox's proportional hazard model. AFP: α-fetoprotein.

[44.1% (113/256)] was the most common in patients without AFP-producing GC (Table 3).

**Comparison of clinicopathological factors and prognosis between AFP-producing GC patients with and without liver metastasis**

We investigated prognostic factors affecting prognosis in AFP-producing GC. Univariate and multivariate analyses using stepwise Cox regression procedures revealed that the presence of liver metastasis was the only independent prognostic factor (HR = 17.6, 95%CI: 2.1-147.1; P = 0.0081) (Table 4). However, no significantly different prognostic factor was observed between patients with or without liver metastasis in AFP-producing GC (Table 5).

**Comparison of survival curves between patients with and without AFP-producing GC according to the presence or absence of liver metastasis**

To confirm that the presence of AFP-producing GC was not an independent prognostic factor in GC, we performed subgroup analysis according to the presence or absence of liver metastasis between patients with and without AFP-producing GC. The results obtained showed that the prognosis of AFP-producing GC was similar to that of AFP-non producing GC according to the presence (Figure 2A, P = 0.3778) or absence (Figure 2B, P = 0.5024) of liver metastasis.

**Table 3** Comparison of recurrence patterns between patients with and without α-fetoprotein -producing gastric cancer n (%)

Site	GC patients with recurrence	
	AFP-producing GC (n = 11)	AFP-non producing GC (n = 256)
Peritoneum	1 (9.1)	113 (44.1)
Liver	10 (90.9)	44 (17.2)
Lung	1 (9.1)	3 (1.2)
Bone	0 (0.0)	13 (5.1)
Lymph nodes	0 (0.0)	48 (18.8)
Others	0 (0.0)	46 (18.0)

The incidence of recurrence was 47.8% in the α-fetoprotein (AFP)-producing group and 20.0% in the AFP-non producing group. The most likely recurrence pattern was liver metastasis and peritoneal metastasis in the AFP-producing group and AFP-non producing group, respectively. GC: Gastric cancer.

**Table 4** Univariate and multivariate survival analyses in α-fetoprotein-producing gastric cancer patients

Factor	Univariate <sup>1</sup>		Multivariate <sup>2</sup>	
	P value	HR	95%CI	P value
Sex				
Male vs female	0.3574			
Age (yr)				
65 ≤ vs < 65	0.0917			
Location				
U vs ML	0.4311			
Macroscopic type				
Type 1/2/3/4	0.3422			
vs Type 0- I / II / III				
Tumor size (mm)				
40 ≤ vs < 40	0.3539			
Histological type				
Undifferentiated vs Differentiated	0.0503			
pT categories				
T2/3/4 vs T1	0.1428			
Venous invasion				
Positive vs negative	0.6696			
Lymphatic invasion				
Positive vs negative	0.6188			
pN categories				
Positive vs negative	0.9855			
Liver metastasis				
Positive vs negative	0.0006	17.587	2.112-147.12	0.0081

<sup>1</sup>The Kaplan-Meier method, and significance was determined by the log-rank test; <sup>2</sup>Multivariate survival analysis was performed using Cox's proportional hazard model.

**DISCUSSION**

AFP-producing GC has been shown to exhibit more aggressive behavior and a poorer prognosis than common GC because of its high incidence of liver metastasis. Liu and his colleagues reported that the 1-, 3- and 5-year survival rates of AFP-producing gastric cancer were 53%, 35%, and 28%, respectively<sup>[10]</sup>. Chang and his colleagues examined 24 patients with AFP-producing gastric cancer and showed that the prognosis was poor due to the high incidence of synchronous and metachronous liver metastasis<sup>[8]</sup>. In Japan, one study reported that the 5-year

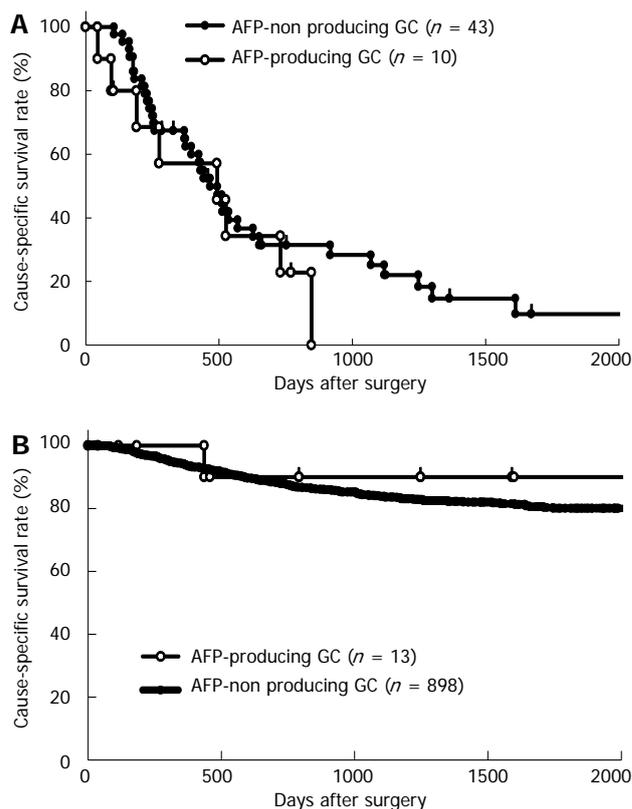
**Table 5 Comparison of clinicopathological factors between  $\alpha$ -fetoprotein-producing gastric cancer patients with and without liver metastasis *n* (%)**

Factor	<i>n</i>	Liver metastasis		<i>P</i> value <sup>1</sup>
		Absence	Presence	
Total	23	13	10	
Sex				0.1203
Male	14	10 (77)	4 (40)	
Female	19	13 (23)	6 (60)	
Age (yr)				0.4173
< 65	9	4 (31)	5 (50)	
≥ 65	14	9 (69)	5 (50)	
Location				0.1717
Upper	4	0 (0)	4 (40)	
Middle	10	7 (54)	3 (30)	
Lower	9	6 (46)	3 (30)	
Macroscopic type				0.4862
Type 0-1 / II / III	2	2 (15)	0 (0)	
Type 1/2/3/4	21	11 (85)	10 (100)	
Histopathological grading				0.2215
Differentiated	10	4 (31)	6 (60)	
Undifferentiated	13	9 (69)	4 (40)	
Tumor size (mm)				0.6036
< 40	4	3 (23)	1 (10)	
≥ 40	19	10 (77)	9 (90)	
Venous invasion				1.0000
Negative	5	3 (23)	2 (20)	
Positive	18	10 (77)	8 (80)	
Lymphatic invasion				0.0886
Negative	7	6 (46)	1 (10)	
Positive	16	7 (54)	9 (90)	
TNM classification				0.7007
pT categories				
T1	4	3 (23)	1 (10)	
T2	3	2 (15)	1 (10)	
T3	7	5 (39)	2 (20)	
T4	9	3 (23)	6 (60)	
pN categories				0.9049
N0	9	5 (39)	4 (40)	
N1	9	4 (31)	5 (50)	
N2	3	2 (15)	1 (10)	
N3	2	2 (15)	0 (0)	
pStage				0.6736
I	3	3 (23)	0 (0)	
II	3	2 (15)	1 (10)	
III	9	5 (39)	4 (40)	
IV	8	3 (23)	5 (50)	

Significantly different values are highlighted using boldface type. <sup>1</sup>*P* values are from  $\chi^2$  or Fisher's exact test and were significant at < 0.05. No significantly different prognostic factor was observed between patients with or without liver metastasis in  $\alpha$ -fetoprotein-producing gastric cancer. TNM: Tumor, nodes, metastasis.

survival rate of AFP-producing GC was 28.4%<sup>[7]</sup>. However, most of these studies have been restricted to overall prognosis in AFP-producing GC. To date, few subgroup analyses have been performed with special reference to liver metastasis and its related prognosis in AFP-producing GC.

In this study, we clearly demonstrated that the clinical behaviors of patients with and without AFP-producing GC were different (Table 1), and also that prognoses between patients with and without AFP-producing GC regarding the presence or absence of liver metastasis



**Figure 2** Survival curves between patients with and without  $\alpha$ -fetoprotein-producing gastric cancer according to the presence (A) or absence (B) of liver metastasis. The prognosis of  $\alpha$ -fetoprotein (AFP)-producing gastric cancer (GC) was similar to that of AFP-non producing GC according to the presence (A, *P* = 0.3778) or absence (B, *P* = 0.5024) of liver metastasis.

were similar in subgroup analyses (Figure 2). At first, we hypothesized that the presence of AFP-producing GC was an independent prognostic factor in GC because the prognosis of AFP-producing GC is known to be poorer than that of common GC. Indeed, the 5-year survival rate of patients with AFP-producing GC was significantly poorer than that of patients without AFP-producing GC. Patients with AFP-producing GC also had a significantly higher incidence of lymph-venous invasion, larger tumor size, lymph node metastasis, liver metastasis, and advanced stage. However, multivariate analysis using step-wise Cox regression procedures demonstrated that the presence of AFP-producing GC was not an independent prognostic factor. These findings suggested that AFP-producing GC is not different from common gastric cancer. AFP-producing GC without liver metastasis does not necessarily have a poor prognosis.

The reason why the prognosis of AFP-producing GC is still poor is that there are currently no standard or recommended treatments for liver metastasis of GC. Even in recent prospective randomized control trials, such as JCOG9912, SPIRITS, TOGA, and START studies using S-1 based regimens, a survival benefit was found particularly in non-measurable tumor recurrences such as peritoneal recurrence<sup>[19-22]</sup>. Therefore, it is important to note that the poorer prognosis of AFP-producing GC

is not related to difficulties in treating it, but from the lack of effective and recommended treatments for liver metastasis of common GC. If a treatment strategy for liver metastasis of GC was established similar to that for colorectal cancer, the prognosis of AFP-producing GC may be markedly improved because of its distinct metastatic pattern.

Concerning patterns of recurrences, distant metastasis and recurrence developed in 47.8% of patients (11/23) with AFP-producing GC and 20.0% of patients (256/1276) without AFP-producing GC. In AFP-producing GC patients, 90.9% of patients (10/11) developed liver metastasis, while peritoneal recurrence occurred in only 9.1% (1/11). In contrast, peritoneal recurrence [44.1% (113/256)] was the most prevalent in patients without AFP-producing GC, with liver metastasis accounting for 17.2% (44/256) (Table 3). Regarding the prognoses of 23 patients with AFP-producing GC, 5-year survival rates between patients with and without liver metastasis were 0% and 90.0%, respectively (data not shown). Moreover, univariate and multivariate analyses in AFP-producing GC revealed that liver metastasis was the only independent prognostic factor (Table 4).

Our results show that liver metastasis should be specifically targeted in an effort to improve the prognosis of AFP-producing GC. However, our study included a small number of patients with AFP-producing GC. Therefore, a larger sample size is needed to confirm these clinical features of AFP-producing gastric cancer.

In the present study, we demonstrated that liver metastasis was the only independent prognostic factor in AFP-producing GC. The absence of standard or recommended treatments for liver metastasis of GC also means that there is no effective treatment strategy for AFP-producing GC. Considering other rarely developing recurrences excluding liver metastasis in AFP-producing GC, the use of hepatic infusion chemotherapy or systemic chemotherapy following resection of hepatic metastasis may be a more efficient treatment than that for liver metastasis of common GC<sup>[23-25]</sup>. In conclusion, clinical behaviors between patients with and without AFP-producing GC were different. However, prognoses were similar according to the presence or absence of liver metastasis. Liver metastasis should be specifically targeted in an effort to improve the prognosis of AFP-producing GC.

## COMMENTS

### Background

$\alpha$ -fetoprotein (AFP)-producing gastric cancer (GC) is generally known to exhibit more aggressive behavior and a poorer prognosis than common GC because of its high incidence of liver metastasis. However, little is known about the clinical behavior of AFP-producing GC with regard to the presence or absence of liver metastasis.

### Research frontiers

The authors re-evaluated the clinicopathological characteristics and clinical outcomes of patients with AFP-producing GC. The results obtained clearly demonstrated that clinical behaviors were different between patients with and without AFP-producing GC. Concerning recurrence, while the incidence of peritoneal recurrence was the highest in AFP-producing GC, liver metastasis was the

most prevalent in AFP-non producing GC. Moreover, the prognosis according to the presence or absence of liver metastasis was similar between patients with and without AFP-producing GC.

### Innovations and breakthroughs

Multivariate analysis revealed that AFP-positivity was not an independent prognostic factor and also that liver metastasis was the only independent prognostic factor in AFP-producing GC. It is important to note that the overall poorer prognosis of AFP-producing GC is not related to difficulties in treating it, but from the lack of effective and recommended treatments for liver metastasis of common GC.

### Applications

The prognosis according to the presence or absence of liver metastasis was similar between patients with and without AFP-producing GC. The results show that liver metastasis should be specifically targeted in an effort to improve the prognosis of AFP-producing GC.

### Terminology

AFP was initially found in the human fetus and is normally produced in the fetal liver and yolk sac. AFP is considered to be a useful tumor marker in screening or monitoring patients with hepatocellular carcinomas or yolk sac tumors. Some studies showed that AFP could be produced in other cancers including primary GC. Liver metastasis refers to cancerous tumors that have spread to the liver from somewhere else in the body. The risk of cancer spreading to the liver depends on the site of the original cancer. Liver metastasis may be present when the original (primary) cancer is diagnosed, or it may occur months or years after the primary tumor is removed.

### Peer review

This is an interesting manuscript from a group that has done excellent work on gastric cancer. The authors evaluated the effect of AFP-producing gastric carcinoma on survival. Their conclusion that AFP production was not an independent predictor of poor survival is in contradistinction to other reports.

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## Clinical outcomes of radiation therapy for early-stage gastric mucosa-associated lymphoid tissue lymphoma

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### Abstract

**AIM:** To evaluate the clinical outcomes of radiation therapy (RT) for early-stage gastric mucosa-associated lymphoid tissue lymphoma (MALToma).

**METHODS:** The records of 64 patients treated between 1998 and 2011 were analyzed retrospectively. For *Helicobacter pylori* (*H. pylori*)-positive patients ( $n = 31$ ), chemotherapy or *H. pylori* eradication therapy was the initial treatment. In patients with failure after *H. pylori* eradication, RT was performed. For *H. pylori*-negative patients ( $n = 33$ ), chemotherapy or RT was the first-line treatment. The median RT dose was 36 Gy. The target volume included the entire stomach and

the perigastric lymph node area.

**RESULTS:** All of the patients completed RT without interruption and showed complete remission on endoscopic biopsy after treatment. Over a median follow-up period of 39 mo, the 5-year local control rate was 89%. Salvage therapy was successful in all relapsed patients. Secondary malignancies developed in three patients. The 5-year overall survival rate was 94%. No patient presented symptoms of moderate-to-severe treatment-related toxicities during or after RT.

**CONCLUSION:** Radiotherapy results in favorable clinical outcomes in patients with early-stage gastric MALToma who experience failure of *H. pylori* eradication therapy and those who are *H. pylori* negative.

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**Key words:** Gastric mucosa-associated lymphoid tissue lymphoma; Radiation therapy; Treatment response

**Core tip:** Radiation therapy is an effective salvage treatment for patients with gastric mucosa-associated lymphoid tissue lymphoma (MALToma) who experience failure of *Helicobacter pylori* (*H. pylori*) eradication therapy. For patients with *H. pylori*-negative gastric MALToma, radiation therapy is recommended as the initial treatment. The risk of treatment-related toxicities and secondary malignancies is acceptable.

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## INTRODUCTION

Mucosa-associated lymphoid tissue lymphoma (MALToma) was first described by Isaacson in 1983<sup>[1]</sup>. The World Health Organization (WHO) and the Revised European American Lymphoma Classification (REAL) later categorized it as an independent entity<sup>[2,3]</sup>.

The stomach is the most common extranodal site of MALToma (50%-70% of all cases), and *Helicobacter pylori* (*H. pylori*) infection is the most important risk factor<sup>[4]</sup>. In numerous studies, *H. pylori* eradication therapy had excellent outcomes for gastric MALToma with a reported complete remission (CR) rate of 70%-80%<sup>[4-6]</sup>. As a result, *H. pylori* eradication therapy is now employed as the sole first-line treatment<sup>[7]</sup>. For *H. pylori*-negative patients, radiation therapy (RT) has recently been accepted as a preferred treatment modality. The role of surgery has declined gradually because of the high incidence of post-operative morbidity and mortality<sup>[8]</sup>.

MALToma is characteristically a localized disease<sup>[9-11]</sup>, and it is very sensitive to radiation. Accordingly, MALToma at extranodal sites, such as the orbital adnexa and the parotid and thyroid glands, has been treated with a moderate dose (25-40 Gy) of RT as an initial treatment<sup>[12-17]</sup>. Retrospective studies have largely confirmed the effectiveness of RT for gastric MALToma<sup>[18-27]</sup>. However, the low case numbers of previous studies render the evidence of the effectiveness of RT insufficient.

In this study, we investigated the clinical outcomes and associated adverse effects of RT in patients with early-stage gastric MALToma who were unresponsive to *H. pylori* eradication therapy and in *H. pylori*-negative patients.

## MATERIALS AND METHODS

Between November 1998 and March 2011, 71 patients who were diagnosed with localized gastric MALToma received RT at Samsung Medical Center. Among them, 7 patients were excluded from this study; 5 patients were excluded because they also had small foci of diffuse large B-cell lymphoma. Two patients were immediately lost to follow up after the completion of RT. We retrospectively analyzed the remaining 64 patients. These patients had stage I or II<sub>1</sub> MALToma according to the Lugano staging system<sup>[28]</sup>. All of the patients were histologically diagnosed with gastric MALToma by endoscopic biopsy. Subsequently, patients underwent a systemic staging work-up, including laboratory tests, such as blood cell counts, biochemical profile and lactate dehydrogenase, computed tomography (CT), positron-emission tomography (PET) or bone marrow biopsy.

### Treatment before RT

Of the 64 patients, 40 patients received treatment related to gastric MALToma before RT. *H. pylori* eradication therapy consisting of a 1-2 wk course of amoxicillin (1000 mg twice daily), clarithromycin (500 mg twice daily) and

a proton pump inhibitor (omeprazole or esomeprazole) followed by maintenance of the proton pump inhibitor was performed in 31 patients. Among the patients treated with *H. pylori* eradication therapy, 3 had no evidence of *H. pylori* infection.

Chemotherapy consisting of either a combination of cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) every 3 wk or cyclophosphamide, vincristine and prednisolone (CVP) every 3 wk was performed in 15 patients until 2002. Among them, 6 patients received both *H. pylori* eradication therapy and chemotherapy (Figure 1).

Twenty-four patients without *H. pylori* infection did not receive any treatment before RT.

### Radiation therapy

All patients underwent simulation using fluoroscopy or a CT simulator. Before simulation, the patients were trained to breathe in a regular and shallow cycle to maintain a constant stomach motion.

The clinical target volume (CTV) was the entire stomach plus the perigastric lymph nodes for stage I gastric MALToma. The CTV for stage II<sub>1</sub> patients was determined as the CTV for stage I gastric MALToma plus a generous margin extending from the involved lymph nodes. To cover the perigastric lymph nodes, we included a 2-cm margin from the outline of stomach wall, which was observed on fluoroscopy after the ingestion of a barium suspension. The planned target volume (PTV) was individualized, considering setup error and stomach movement. CT planning was conducted for 39 patients following the introduction of the CT simulator. The entire stomach was defined at every respiratory stage in 4-dimensional CT slices. After the internal target volume (ITV) was defined, the CTV was set as the ITV plus a 1-cm margin to cover the perigastric lymph nodes. The PTV was the CTV plus 1 cm, and the planning geometry consisted of 2 to 4 coplanar or non-coplanar beams using high-photon energy (10-15 MV).

The daily fraction size was either 1.8 Gy or 2.0 Gy. The total radiation dose ranged from 30 Gy to 44 Gy, with a median of 36 Gy.

### Treatment response

We assessed the treatment response histologically using endoscopic biopsy after the completion of RT. The first post-RT endoscopic examination was typically performed 1-2 mo after RT. The next endoscopic biopsies were performed every 3 to 6 mo in the first two or three years and annually thereafter. CT findings were not considered to evaluate the treatment response. The response criteria were based on the GELA (Groupe d'Etude des Lymphomes de l'Adulte) histologic grading system<sup>[29]</sup>.

### Statistical analysis

The end points were overall survival (OS) and local control (LC). Time was calculated from the initiation of RT to the event of interest. LC was defined as the time in-

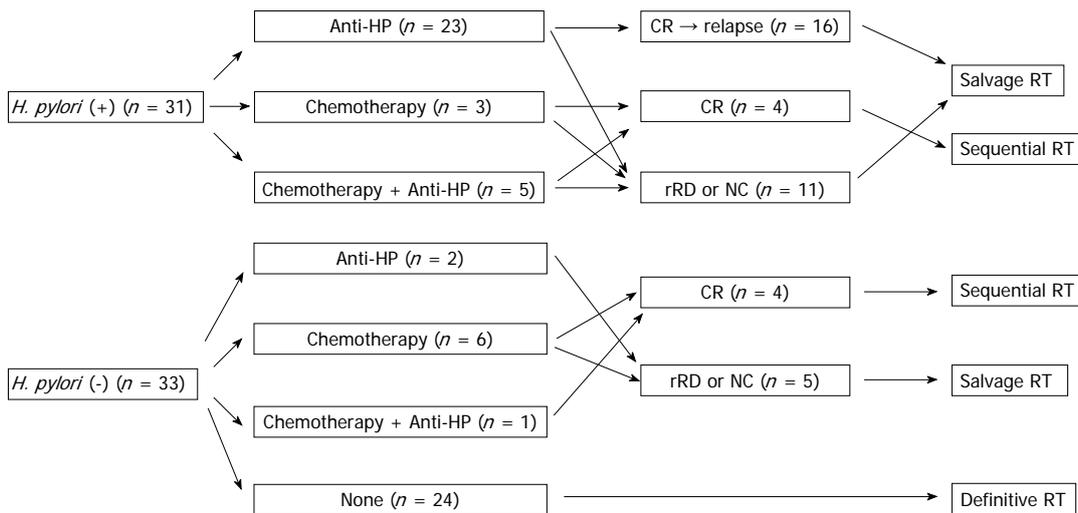


Figure 1 Initial treatment before radiation therapy. *H. pylori*: *Helicobacter pylori*; Anti-HP: anti-*Helicobacter pylori* therapy; CR: Complete remission; rRD: Residual responding disease; NC: No change; RT: Radiation therapy.

Table 1 Patient characteristics n (%)	
Characteristic	No. of patients (n = 64)
Gender	
Male	38 (59)
Female	26 (41)
Age (yr)	Median, 49 Range, 24-75
Lugano staging system	
I	53 (83)
II <sub>1</sub>	11 (17)
Disease location	
Cardia	2 (3)
Fundus	5 (8)
Body	35 (55)
Antrum	7 (11)
Diffuse	15 (23)
<i>H. pylori</i> infection	
Negative	33 (52)
Positive	31 (48)
Prior treatment	
<i>H. pylori</i> eradication	25 (39)
Chemotherapy	9 (14)
Combined	6 (9)
None	24 (38)
Response to prior treatment	
Complete histologic remission (CR)	17
Responding residual disease (rRD)	11
No change (NC)	12
Not evaluable	24
Radiation dose	Median, 36 Gy
≥ 40 Gy	18 (28)
36 Gy	38 (59)
30 Gy	8 (13)
RT technique	
AP/PA	25 (39)
3-D conformal	39 (61)

*H. pylori*: *Helicobacter pylori*; RT: Radiation therapy; AP/PA: Anteroposterior/posteroanterior.

terval to relapse, which was confirmed by biopsy. OS was defined as the period from diagnosis to the date of last

follow up or death. We calculated the LC and OS rates using the Kaplan-Meier method. All analyses were performed using IBM SPSS Statics version 19.0 (SPSS Inc., Chicago, IL).

## RESULTS

### Patient characteristics

Patient characteristics are summarized in Table 1. The median age was 49 years, and 53 patients (83%) were stage I. The gastric body was the site most commonly involved, and *H. pylori* infection was detected in 31 patients (48%). As an initial therapy, 31 patients (48%) received *H. pylori* eradication therapy, and 24 patients (38%) received RT.

Seventeen of 40 patients (43%) achieved CR with prior treatment before RT. Nine patients who achieved CR with *H. pylori* eradication therapy relapsed after a median disease-free interval of 19 mo (range: 9 to 56 mo). CR after chemotherapy was achieved in 5 of 9 patients. The combination of *H. pylori* eradication therapy and chemotherapy was successful in 3 patients. Those who achieved CR after chemotherapy received RT as sequential therapy. The remaining 23 patients had persistent disease at the time of referral for salvage RT (Figure 1).

The median interval between the initiation of first treatment and the initiation of RT was 8 mo (range, 1 to 40 mo).

### RT response

All patients showed a histological CR at the post-treatment endoscopic biopsy. Three patients had not achieved CR at the first post-treatment endoscopic biopsy, but they had achieved a histological CR at a subsequent evaluation 4 to 5 mo after RT without any further treatment.

### Survival

Over a median follow-up period of 39 mo (range, 9-131

**Table 2** Patients with local recurrence after achieving complete remission with radiation therapy

	Initial <i>H. pylori</i>	Initial Tx before RT	DFI (mo)	<i>H. pylori</i> at relapse	Tx after relapse	Final response
Case 1	+	Anti-HP	14	-	Observation	CR
Case 2	-	None	11	-	Observation	CR
Case 3	-	None	26	-	Observation	CR
Case 4	-	None	20	+	Anti-HP	CR
Case 5	-	None	38	+	Anti-HP	CR

*H. pylori*: *Helicobacter pylori*; anti-HP: anti-*H. pylori* antibiotics; CR: Complete remission; DFI: Disease-free interval; Tx: Treatment.

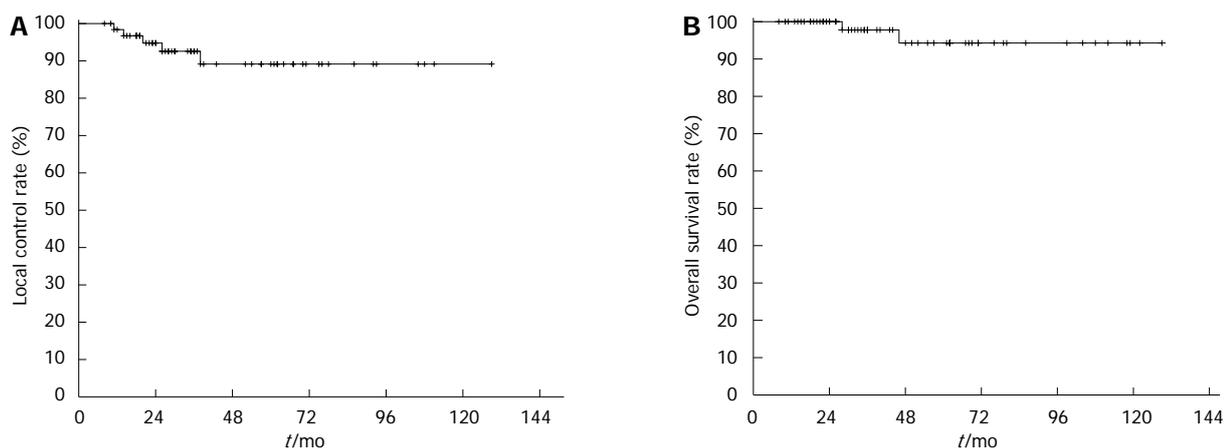


Figure 2 Local control rate (A) and overall survival rate (B) for all patients.

mo), MALToMa relapsed in 5 patients (Table 2), and the 5-year LC of all patients was 89% (Figure 2A). Two patients (cases 4 and 5 in Table 2) received *H. pylori* eradication therapy due to positive conversion of *H. pylori* at relapse and finally gained secondary CR. The other three patients (cases 1, 2 and 3) who were not *H. pylori* infected at relapse did not receive any treatment, and MALToMa was not detected on subsequent endoscopy.

At the study end point, 62 patients remained alive. One patient died due to relapse following the development of malignant lymphoma involving the mesenteric lymph nodes and bone marrow at 29 mo after RT. Another patient died of intercurrent disease 47 mo after RT. The 5-year overall survival rate was 94% (Figure 2B).

#### Treatment-related toxicities and secondary malignancies

All patients completed RT without interruptions due to treatment-related toxicities. Some patients experienced mild nausea and epigastric soreness, but these symptoms were managed with anti-emetics or antacids. None of the patients complained of gastrointestinal bleeding or perforations. Neither renal nor hepatic toxicities developed.

One patient with *H. pylori* infection was diagnosed with metachronous gastric cancer 21 mo after RT. He underwent subtotal gastrectomy with Billroth type I reconstruction. On pathologic examination, tubular adenocarcinoma was confined to the mucosa, and metastasis to the regional lymph nodes was not found.

Transformation into malignant lymphoma occurred in two patients at intervals of 26 and 31 mo following

the initiation of RT. The sites involved were the mesenteric lymph node and the supraclavicular lymph node, respectively. These patients received 6 cycles of salvage chemotherapy with the CHOP regimen. One patient died of rapid disease progression 6 mo later. The other patient survived in a cured state.

## DISCUSSION

Currently, *H. pylori* eradication therapy is the only established treatment for low-grade gastric MALToMa in *H. pylori*-positive patients<sup>[7]</sup>. The treatment modality that should be selected for patients who do not respond to *H. pylori* eradication therapy is currently controversial. Recently, many small retrospective studies and a pooled-data analysis<sup>[19,21,22,26,30]</sup> have demonstrated that RT has excellent clinical outcomes and feasibility. As a result, RT has become a preferred non-invasive local treatment modality. The results of our present study also support the effectiveness of RT for these patients.

The application of salvage RT in this setting depends to a high degree on defining the time at which *H. pylori* eradication failed. Most studies with salvage RT for gastric MALToMa have not defined the time to *H. pylori* eradication therapy failure. Our institution also did not have consensus regarding the failure of *H. pylori* eradication therapy. The range of the interval between the initiation of prior treatment and the timing of RT initiation was variable and not homogeneous. Previous studies have demonstrated that 80%-90% of patients may achieve a

histological CR within one year after *H. pylori* eradication therapy<sup>[6,20]</sup>. The European Society of Medical Oncology has proposed that at least 12 mo be allowed before initiating another treatment in patients who achieve clinical and endoscopic remission in addition to *H. pylori* eradication, albeit with persistent lymphoma at the histological level<sup>[7]</sup>.

In addition, reference data for the evaluation of RT response are lacking. In this study, 3 patients did not show CR at the first post-treatment endoscopy, which was performed 1 to 2 mo after RT, but they had achieved CR at the next endoscopy, which was performed 4 to 5 mo later. We therefore suggest that a minimum follow-up period of 6 mo is needed to evaluate the effects of RT.

For *H. pylori*-negative patients, *H. pylori* eradication therapy can be attempted as an initial treatment because of the possibility of a false-negative test or low bacteria counts. However, the response rate has been reported to vary (0%-83%)<sup>[21,27,31]</sup>. In contrast, several small retrospective studies reported a response rate of 100% for RT as the initial treatment for *H. pylori*-negative patients with MALToma<sup>[18,21,25,27]</sup>. In these previous studies, CR was confirmed in all patients at the post-treatment endoscopic biopsy after RT. In the absence of randomized trial data due to the rarity of disease, we cannot directly compare *H. pylori* eradication therapy with RT as an initial treatment for *H. pylori*-negative patients. However, a survey of the literature suggests that RT shows superior performance compared to *H. pylori* eradication therapy in the initial treatment of *H. pylori*-negative patients. Recent evidence indicates that the t(11;18)(q21;q21) translocation in gastric MALToma predicts resistance to *H. pylori* eradication therapy<sup>[32]</sup>. This same translocation has been detected in nearly half of patients with *H. pylori*-negative gastric MALToma<sup>[33]</sup>. Therefore, in these subgroups, RT may be an appropriate treatment. As additional data become available, routine testing for the t(11;18)(q21;q21) translocation at the time of MALToma diagnosis may become a useful guide for selecting the initial treatment.

The 5-year LC rate in this study was 89%, which is comparable to those reported by previous studies<sup>[13,17,18]</sup>. Until 6 mo after the completion of RT, all patients showed histological CR on follow-up endoscopic biopsy. However, 5 patients developed relapse at disease-free intervals of 11 to 38 mo. Three patients achieved histological CR on the subsequent evaluation without any further treatment, which could be due to false-positive results at the initial examinations. The other two patients, who were initially *H. pylori* negative, developed recurrence coincident with *H. pylori* infection, and they were successfully treated with *H. pylori* eradication therapy as a salvage treatment. This example may indicate an important role for *H. pylori* infection in the mechanism of the local recurrence of gastric MALToma.

The major concern regarding stomach irradiation is the risk of perforation and bleeding. A collective review places this risk at 4% or less<sup>[34]</sup>. Additionally, the risk may be much lower in early-stage and low-grade disease, given

the lower radiation doses and target volumes. Another concern is renal toxicity. In the past, the irradiated target volume was the entire abdomen followed by an additional dose to the entire stomach and the perigastric lymph nodes<sup>[24]</sup>. With increasing knowledge of the spread pattern of low-grade gastric MALToma, the target volume has been reduced to the stomach plus the regional lymphatics<sup>[11]</sup>. Local RT with the use of three-dimensional conformal or intensity-modulated techniques has the particular advantage of reducing the radiation dose to the kidneys, particularly on the left side<sup>[19,35]</sup>. Our study, in agreement with numerous other studies, demonstrated no gastric perforation or bleeding and no renal toxicity attributable to RT.

Whether secondary malignancies such as gastric cancer develop years after irradiation is still controversial. Reports of secondary malignancies within the RT field in the treatment of gastric MALToma appear to be rare<sup>[13,21,23]</sup>. In this study, secondary gastric cancer occurred in one case. For secondary gastric cancer, gastric MALToma itself could be an additional risk factor because of the common pathogenesis, *i.e.*, *H. pylori* infection, irrespective of the treatment modality<sup>[34,36-38]</sup>. However, the effects of irradiation cannot be neglected. Because the latent period from irradiation to the development of secondary malignancies can reach a few decades and nearly all patients with gastric MALToma are expected to be long-term survivors in a cured state, close, long-term follow up is required to observe the development of radiation-induced secondary cancer.

Malignant transformation in MALToma is uncommon. The transformation rates reported in some studies ranged from 3% to 19% and did not apparently depend on the site involved<sup>[10,14,17]</sup>. In the present study, transformation developed in 2 patients (3.1%) with a disease-free interval of 26 to 31 mo, which is consistent with other published reports (ranging from 6 to 116 mo)<sup>[14,17]</sup>. Following malignant transformation, the mortality rate is higher than that for MALT lymphoma.

In conclusion, the results of the present study further support the use of RT for patients with MALToma who experience failure of *H. pylori* eradication therapy and for *H. pylori*-negative patients. The rationale of treating such patients with RT can be summarized as follows. First, gastric MALToma tends to be a localized disease. Second, MALToma is a radio-sensitive tumor. Third, RT has the advantage of stomach preservation. Lastly, RT has low treatment-related morbidity. Although local relapse developed in some cases, all cases were salvaged successfully. Ongoing risks for secondary malignancy or malignant transformation warrant regular long-term follow-up with systemic evaluation and testing for *H. pylori* infection.

## COMMENTS

### Background

Gastric mucosa-associated lymphoid tissue lymphoma (MALToma) is highly associated with *Helicobacter pylori* (*H. pylori*) infection. Therefore, *H. pylori* eradication therapy is the standard treatment. However, in clinical practice,

persistent disease or recurrence after *H. pylori* eradication therapy are often found. Additionally, a substantial number of gastric MALToma patients are not *H. pylori* positive. Radiation therapy (RT) has been shown to have good clinical outcomes and feasibility for the treatment of gastric MALToma in these patients in many small retrospective studies.

### Research frontiers

Some reports from single institutions have shown that RT is effective for patients with gastric MALToma that is resistant to *H. pylori* eradication therapy or that recurs following an initial clinical response. Additionally, in other small reports, patients with *H. pylori*-negative gastric MALToma and low response rates to *H. pylori* eradication therapy were treated successfully with RT.

### Innovations and breakthroughs

This study is one of the largest single-institution experiences to report the clinical outcomes of gastric MALToma treated with RT. RT has an excellent role as either a salvage or definitive treatment for gastric MALToma. In particular, for *H. pylori*-negative gastric MALToma patients, in whom the t(11;18)(q21;q21) translocation is frequently found, RT should be applied as the initial treatment because this translocation tends to be resistant to *H. pylori* eradication therapy. With aid of the three-dimensional conformal technique, fatal toxicities related to radiation can be minimized.

### Applications

RT is the preferred salvage treatment modality for *H. pylori* infection-associated gastric MALToma patients who are unresponsive to *H. pylori* eradication therapy. Additionally, it can be an effective initial treatment for gastric MALToma patients without *H. pylori* infection.

### Terminology

MALToma is a B cell-originating cancer in the marginal zone of the MALT, which is the diffuse system of small areas of lymphoid tissue found in various sites of the body.

### Peer review

This paper is well written. It is acceptable for publication.

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## Glycyrrhizinate reduces portal hypertension in isolated perfused rat livers with chronic hepatitis

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### Abstract

**AIM:** To investigate the effects of diammonium glycyrrhizinate (Gly) on portal hypertension (PHT) in isolated portal perfused rat liver (IPPL) with carbon tetrachloride (CCl<sub>4</sub>)-induced chronic hepatitis.

**METHODS:** PHT model was replicated with CCl<sub>4</sub> in rats for 84 d. Model was identified by measuring the ascetic amounts, hepatic function, portal pressure *in vivo*, splenic index, and pathological alterations. Inducible nitric oxide synthase (iNOS) in liver was assessed by immunohistochemistry. IPPLs were performed at d<sub>0</sub>, d<sub>28</sub>, d<sub>56</sub>, and d<sub>84</sub>. After phenylephrine-induced constriction, Gly was geometrically used to reduce PHT.

Gly action was expressed as median effective concentration (EC<sub>50</sub>) and area under the curve (AUC). Underlying mechanism was exploited by linear correlation between AUC values of Gly and existed iNOS in portal triads.

**RESULTS:** PHT model was confirmed with ascites, splenomegaly, serum biomarkers of hepatic injury, and elevated portal pressure. Pathological findings had shown normal hepatic structure at d<sub>0</sub>, degenerations at d<sub>28</sub>, fibrosis at d<sub>56</sub>, cirrhosis at d<sub>84</sub> in PHT rats. Pseudo lobule ratios decreased and collagen ratios increased progressively along with PHT development. Gly does dose-dependently reduce PHT in IPPLs with CCl<sub>4</sub>-induced chronic hepatitis. Gly potencies were increased gradually along with PHT development, characterized with its EC<sub>50</sub> at  $2.80 \times 10^{-10}$ ,  $3.03 \times 10^{-11}$ ,  $3.77 \times 10^{-11}$  and  $4.65 \times 10^{-11}$  mol/L at d<sub>0</sub>, d<sub>28</sub>, d<sub>56</sub> and d<sub>84</sub>, respectively. Existed iNOS was located at hepatocyte at d<sub>0</sub>, stellate cells at d<sub>28</sub>, stellate cells and macrophages at d<sub>56</sub>, and macrophages in portal triads at d<sub>84</sub>. Macrophages infiltrated more into portal triads and expressed more iNOS along with PHT development. AUC values of Gly were positively correlated with existed iNOS levels in portal triads.

**CONCLUSION:** Gly reduces indirectly PHT in IPPL with CCl<sub>4</sub>-induced chronic hepatitis. The underlying mechanisms may relate to rescue NO bioavailability from macrophage-derived peroxynitrite in portal triads.

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**Key words:** Chronic hepatitis; Portal hypertension; Isolated portal perfused rat liver; Diammonium glycyrrhizinate; Inducible nitric oxide synthase

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## INTRODUCTION

Portal hypertension (PHT) is a fetal complication in patients with advanced chronic hepatitis<sup>[1]</sup>. Some changes are reversible in PHT pathogenesis, such as an increase of hepatic vascular resistance and an elevation of portal hyperemia<sup>[2]</sup>. Therefore, it is possible to develop drugs for PHT therapy.

Insufficient intrahepatic nitric oxide (NO) bioavailability is involved in PHT development<sup>[3]</sup>. Macrophages in portal triads generate peroxynitrite *via* inducible nitric oxide synthase (iNOS)<sup>[4]</sup> and superoxide *via* NADPH oxidases<sup>[5]</sup>. Under chronic hepatitis, macrophages infiltrated more into portal triads and expressed more iNOS<sup>[6,7]</sup>. Peroxynitrite was derived from NO and superoxide, to reduce NO bioavailability<sup>[3,4]</sup>. The decreased NO availability leads to an increase in intrahepatic portal resistance, resulting in PHT<sup>[3,4]</sup>.

Diammonium glycyrrhizinate (Gly), a molecule derived from a medical plant of *Radix glycyrrhizae*, is effective in treatment of PHT patients<sup>[8]</sup> and animals<sup>[9,10]</sup>, but the underlying mechanisms are still unclear<sup>[9-11]</sup>. In our previous studies, we demonstrated that Gly lower PHT in isolated portal perfused rat livers (IPPRLs) at physiological status<sup>[12]</sup>.

Suitable perfuse velocities were designated as anatomic preloads in IPPRLs with CCl<sub>4</sub>-induced chronic hepatitis at four stages<sup>[13]</sup>. A pharmacodynamic model of PHT had been developed in IPPRLs with chronic hepatitis, as median effective concentration (EC<sub>50</sub>) values of phenylephrine and acetylcholine<sup>[14]</sup>. This model makes it possible to evaluate candidates for PHT therapy. Furthermore, several studies have shown that Gly enhances NO generation from inflammatory macrophages<sup>[15,16]</sup>, limits superoxide release and increases NO bioavailability<sup>[16]</sup>.

In this study, we investigated the effects of Gly on PHT in the IPPRLs with CCl<sub>4</sub>-induced chronic hepatitis, and on NO bioavailability from the macrophages in portal triads.

## MATERIALS AND METHODS

### Animals and materials

Male Wistar rats (200 ± 13 g) were obtained from Animal Centre of Chinese Academy of Medical Sciences. Rats were maintained in a Special Pathogen Free laboratory, with a 25.0 ± 0.2 °C, a 12-h/12-h light/dark photoperiod and 45% ± 2% humidity. All rats were fed standard rodent pellets and allowed free access to filtered water. All experiment procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China.

Carbon tetrachloride (CCl<sub>4</sub>, CAS 56-23-5), olive oil (CAS 8001-25-0) and heparin sodium (MW 12000, CAS 9041-08-1) were obtained from Sinopharm Chemical Reagent Company; Acetylcholine chloride (CAS 60-31-1) and phenylephrine hydrochloride (CAS 61-76-7) were from Sigma (United States); Diammonium Gly (CAS 79165-06-3) were from Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

### Portal hypertensive model

As described previously<sup>[13,14,17]</sup>, PHT model was replicated by subcutaneous injection with 3 mL/kg mixture of 40% (v/v) CCl<sub>4</sub> in olive oil, twice weekly for 0, 4, 8 and 12 wk; age-matched animals did with olive oil along as vehicle control (Figure 1). Forty-eight hours after last injection, rat was weighted (W<sub>b</sub>) and anesthetized with pentobarbital sodium (50 mg/kg) subcutaneously, a midline incision was made to open abdominal cavity, soak up ascitic samples, expose liver vessels, measure portal pressure, collect blood sample, and canalize hepatic artery, portal vein, hepatic vein. The remained blood in the isolated livers was eliminated with perfusate containing 20.0 µg/mL of heparin sodium through hepatic artery.

### Isolated perfused liver system

As described previously<sup>[13,14,17]</sup>, the velocity in each IPPRL was finely controlled by a quantified roller pump. The perfuse pressure was continuously recorded with the Powerlab linked to a computer with a pressure transducer immediately ahead of the portal inlet cannula. The global viability of portal perfused livers was assessed with gross appearance, a stable perfusate pH (7.40 ± 0.10), a stable perfuse pressure, and active response to acetylcholine.

### Ascitic quantification

As described previously<sup>[13,14,17]</sup>, exudative liquid was soaked up by dried soft paper in a tube (W<sub>0</sub>). Before (W<sub>1</sub>) and after (W<sub>2</sub>) the wet paper with exudation was dried enough, the paper with tube was weighed exactly. Basing on the body weight (W<sub>b</sub>), exuded water (EWR) and dried mass ratios (EDMR) were calculated as  $EWR = (W_1 - W_2)/W_b \times 1000$  and  $EDMR = (W_2 - W_0)/W_b \times 1000$ , expressed as exudative weight (g) per kilogram body weight (g/kg).

### Hepatic function

Sera were separated by centrifugation at 300 g for 5 min and were stored at -80 °C. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP) and albumin (Alb) were determined by an autoanalyzer (Hitachi 7060; Hitachi, Japan) with commercial kits (Biosino Biotechnology and Science, China) according to the manufacturer's instructions.

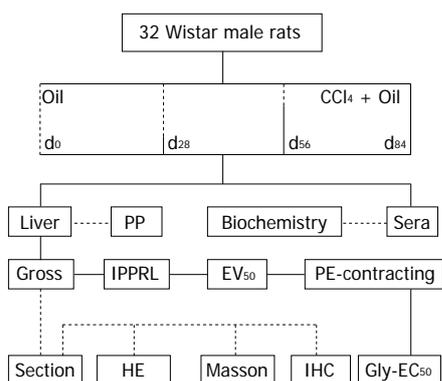
### Pathological changes

**Organ indexes:** Liver, spleen and kidneys were weighed (W<sub>i</sub>) and the organ indexes (OI) were calculated for each

**Table 1 Exudations and organ indexes (mean ± SE, n = 8)**

Groups	Exudations [g/(100g)]		Organ indexes [g/(100g)]		
	Water	Dried mass	Liver	Spleen	Kidneys
d <sub>00</sub>	0.223 ± 0.091	0.490 ± 0.260	2.801 ± 0.347	0.163 ± 0.040	0.731 ± 0.096
d <sub>28</sub>	0.372 ± 0.127 <sup>b</sup>	1.250 ± 0.210 <sup>b</sup>	5.936 ± 1.081 <sup>b</sup>	0.236 ± 0.037 <sup>b</sup>	0.854 ± 0.095 <sup>a</sup>
d <sub>56</sub>	0.791 ± 0.134 <sup>bd</sup>	1.540 ± 0.150 <sup>bd</sup>	4.472 ± 0.909 <sup>bd</sup>	0.292 ± 0.103 <sup>b</sup>	0.861 ± 0.117 <sup>a</sup>
d <sub>84</sub>	2.267 ± 0.732 <sup>bdf</sup>	3.590 ± 1.610 <sup>bdf</sup>	3.037 ± 0.349 <sup>df</sup>	0.409 ± 0.095 <sup>bde</sup>	1.071 ± 0.117 <sup>bdf</sup>

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs d<sub>00</sub>; <sup>d</sup>P < 0.01 vs d<sub>28</sub>; <sup>e</sup>P < 0.05, <sup>f</sup>P < 0.01 vs d<sub>56</sub>.



**Figure 1 Experimental design of glycyrrhizinate on portal hypertension.** Rat model of portal hypertension was replicated with CCl<sub>4</sub> in olive oil (full line), age-matched rats did with olive oil (dotted line). PP: Portal pressure in vivo; IPPRL: Isolated portal perfused rat liver; EV<sub>50</sub>: Median effective velocity; PE: Phenylephrine; HE: Haematoxylin-eosin stain; Masson: Masson's trichrome stain; IHC: Immunohistochemistry; Gly: Diammonium glycyrrhizinate; EC<sub>50</sub>: Median effective concentration of Gly relaxation.

rat (OI<sub>i</sub> = W<sub>i</sub>/W<sub>b</sub> × 100).

**Histological morphometry:** After perfusion as described previously<sup>[13,14]</sup>, a portion of left lobe from each liver was fixed in 10% buffered formaldehyde, embedded in paraffin, cut as 6 μm sections, and stained with haematoxylin-eosin (HE) and Masson's trichrome (Masson). Images were acquired with a Digital Pathology system (Hamamatsu, Japan). Ten fields from each liver were randomly selected, the percentage of lobules (ratio of lobule area per total analyzed field area × 100, under × 10, in HE) and one-ten thousandth of collagen (ratio of collagen area per total analyzed field area × 10000, under × 40, in Masson) were measured using Image Pro Plus analysis system 7.0.1 (No41N70000-60555, Media cybernetics, United States); the average of values from ten random fields generated a datum.

**Immunohistochemical morphometry:** Formalin-fixed, paraffin-embedded, 6 μm sections were used. Rat iNOS was stained with a rabbit polyclonal antibody (1:200, Santa Cruz Biotechnology) and an avidin-biotin peroxidase immunostaining kit with diaminobenzidine (Boster, Wuhan, China). One set of sections was counterstained with hematoxylin for observing cellular location; the other set did not for quantifying existed levels. Microscopic images under × 40 were acquired with Digital Pathology System.

Mean optical density (OD), positive staining area (A<sub>P</sub>) and total observed area (A<sub>T</sub>) were measured using Image Pro Plus Analysis System. Levels of existed iNOS were represented as diaminobenzidine-OD average per volume [OD × (A<sub>P</sub>/A<sub>T</sub>)<sup>3/2</sup>]; the average of values from ten random fields generated a datum.

**Pharmacodynamic actions**

As described previously<sup>[13,14]</sup>, pharmacodynamical model in IPPRLs with chronic hepatitis was modified delicately with structural and functional preloads at d<sub>0</sub>, d<sub>28</sub>, d<sub>56</sub> or d<sub>84</sub> in PHT development. Perfused velocity as structural preload was defined as 3935.50, 4720.63, 4753.35, or 5164.16 μL/min<sup>[13]</sup>; Concentration of phenylephrine as functional preloads was designated as 1.69 × 10<sup>-10</sup>, 2.64 × 10<sup>-10</sup>, 5.82 × 10<sup>-10</sup> or 8.24 × 10<sup>-10</sup> mol/L<sup>[14]</sup>.

Portal pressure in each IPPRL was initially maintained at defined velocity for 30 min<sup>[13]</sup>. Maintained pressure was near to portal pressure *in vivo*. Elevated pressure was stabilized for 10 min after phenylephrine-induced constriction at designated concentration<sup>[14]</sup>. Elevated pressure was considered as the baseline for analyzing Gly to relax hepatic portal venules. Cumulative geometric concentrations of Gly (10<sup>-12</sup>-10<sup>-6</sup> mol/L, k = 0.10) were finally used in recirculating perfusate to reduce elevated portal pressure. Gly concentration-response curve was regressed from the cumulative concentrations and the changed percentage of perfused pressure from the baseline of phenylephrine constriction.

**Statistical analysis**

Data are expressed as mean ± SD in each stage. Unpaired *t* test was used, P < 0.05 was considered significance. Equation, EC<sub>50</sub> with its 95%CI and area under the curve (AUC) of Gly were calculated by regression analysis using Graph-Pad Prism 4 (Graph-Pad Software) in non-linear fit and various slopes. EC<sub>50</sub> values of Gly were regressed linearly with the duration (0, 4, 8 and 12 wk) in chronic hepatitis; AUC values of Gly did with the levels of existed iNOS in portal triads.

**RESULTS**

**General feature**

**Ascites:** As one of PHT consequences, exuded watery and dried mass ratios increased progressively along with PHT development (P < 0.01, Table 1).

**Table 2** Changed levels of serum biomarkers (mean ± SE, n = 8)

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	TP (g/L)	Alb (g/L)
d <sub>0</sub>	49.63 ± 14.03	124.13 ± 20.20	72.88 ± 14.58	63.63 ± 4.26	33.12 ± 1.97
d <sub>28</sub>	237.13 ± 107.91 <sup>b</sup>	503.25 ± 235.12 <sup>b</sup>	318.75 ± 147.81 <sup>b</sup>	56.84 ± 5.43 <sup>b</sup>	25.93 ± 3.16 <sup>b</sup>
d <sub>56</sub>	160.25 ± 42.39 <sup>bc</sup>	411.63 ± 143.51 <sup>b</sup>	363.50 ± 170.36 <sup>b</sup>	54.91 ± 5.27 <sup>b</sup>	24.13 ± 4.25 <sup>b</sup>
d <sub>84</sub>	230.00 ± 58.58 <sup>bf</sup>	475.50 ± 201.02 <sup>b</sup>	303.00 ± 225.94 <sup>b</sup>	58.36 ± 7.92	26.98 ± 6.86 <sup>a</sup>

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs d<sub>0</sub>; <sup>c</sup>P < 0.05 vs d<sub>28</sub>; <sup>f</sup>P < 0.01 vs d<sub>56</sub>. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TP: Total protein; Alb: Albumin.

**Table 3** Pathological changes and inducible nitric oxide synthase levels (mean ± SE, n = 8)

Groups	PP (mmHg)	Lobule	Collagen	iNOS-OD/V
d <sub>0</sub>	6.648 ± 2.235	0.564% ± 0.022%	0.048% ± 0.013%	0.165 ± 0.011
d <sub>28</sub>	9.225 ± 2.114 <sup>b</sup>	0.511% ± 0.031% <sup>b</sup>	0.248% ± 0.120% <sup>b</sup>	0.197 ± 0.005 <sup>b</sup>
d <sub>56</sub>	24.724 ± 3.368 <sup>bd</sup>	0.230% ± 0.024% <sup>bd</sup>	1.974% ± 0.637% <sup>bd</sup>	0.132 ± 0.010 <sup>bd</sup>
d <sub>84</sub>	26.752 ± 3.263 <sup>bdf</sup>	0.134% ± 0.009% <sup>bdf</sup>	5.925% ± 1.761% <sup>bdf</sup>	0.236 ± 0.040 <sup>bdf</sup>

<sup>b</sup>P < 0.01 vs d<sub>0</sub>; <sup>d</sup>P < 0.01 vs d<sub>28</sub>; <sup>f</sup>P < 0.01 vs d<sub>56</sub>. PP: Portal pressure; iNOS: Inducible nitric oxide synthase.

**Organ indexes:** Hepatic indexes were the lowest at d<sub>0</sub>, the highest at d<sub>28</sub>, higher at d<sub>56</sub> and lower at d<sub>84</sub>. Splenic or renal indexes increased gradually from d<sub>0</sub> to d<sub>84</sub> (P < 0.01, Table 1).

**Hepatic function:** ALT and AST activities in sera at d<sub>28</sub> increased from that at d<sub>0</sub>, then relieved at d<sub>56</sub> and d<sub>84</sub>. ALP activities at d<sub>28</sub> and d<sub>56</sub> increased from that at d<sub>0</sub>, then relieved at d<sub>84</sub>; TP and Alb levels at d<sub>28</sub> and d<sub>56</sub> decreased from that at d<sub>0</sub>, then relieved at d<sub>84</sub> (P < 0.05, Table 2).

**Portal hypertensive feature**

**PHT:** The portal pressures in vivo increased progressively from d<sub>0</sub> to d<sub>84</sub> in PHT development. Elevated portal pressures (> 20 mmHg) at d<sub>56</sub> and d<sub>84</sub> reached to the diagnostic criteria of clinic PHT (P < 0.01, Table 3).

**Pathologic changes:** At d<sub>0</sub>, histological observation showed normal hepatic structure; some droplet steatosis appeared in hepatocytes, collagen located only at portal triads (Figure 2A). At d<sub>28</sub>, hepatic steatosis and cellular swellings were observed pathologically; hepatic sinusoids were severely narrowed by enlarged hepatic cords (Figure 2B). At d<sub>56</sub>, hepatic fibrosis had been demonstrated with more deposited collagen, relieved enlarged hepatic cords, and widened hepatic sinusoids. Deposited collagen had extended into lobules from portal triads, separated lobules incompletely; therefore circulative directions had not changed in hepatic sinusoids (Figure 2C). At d<sub>84</sub>, hepatic cirrhosis had been demonstrated with deposited collagen separated through the lobules, pseudo lobules formed all over, circulative directions had completely deranged in hepatic sinusoids (Figure 2D).

**Pseudo lobule ratio:** Pseudo lobule ratios decreased progressively from d<sub>0</sub> to d<sub>84</sub> in PHT development (P < 0.01, Table 3).

**Collagen ratio:** Collagen ratios increased progressively from d<sub>0</sub> to d<sub>84</sub> in PHT development (P < 0.01, Table 3).

**iNOS distribution**

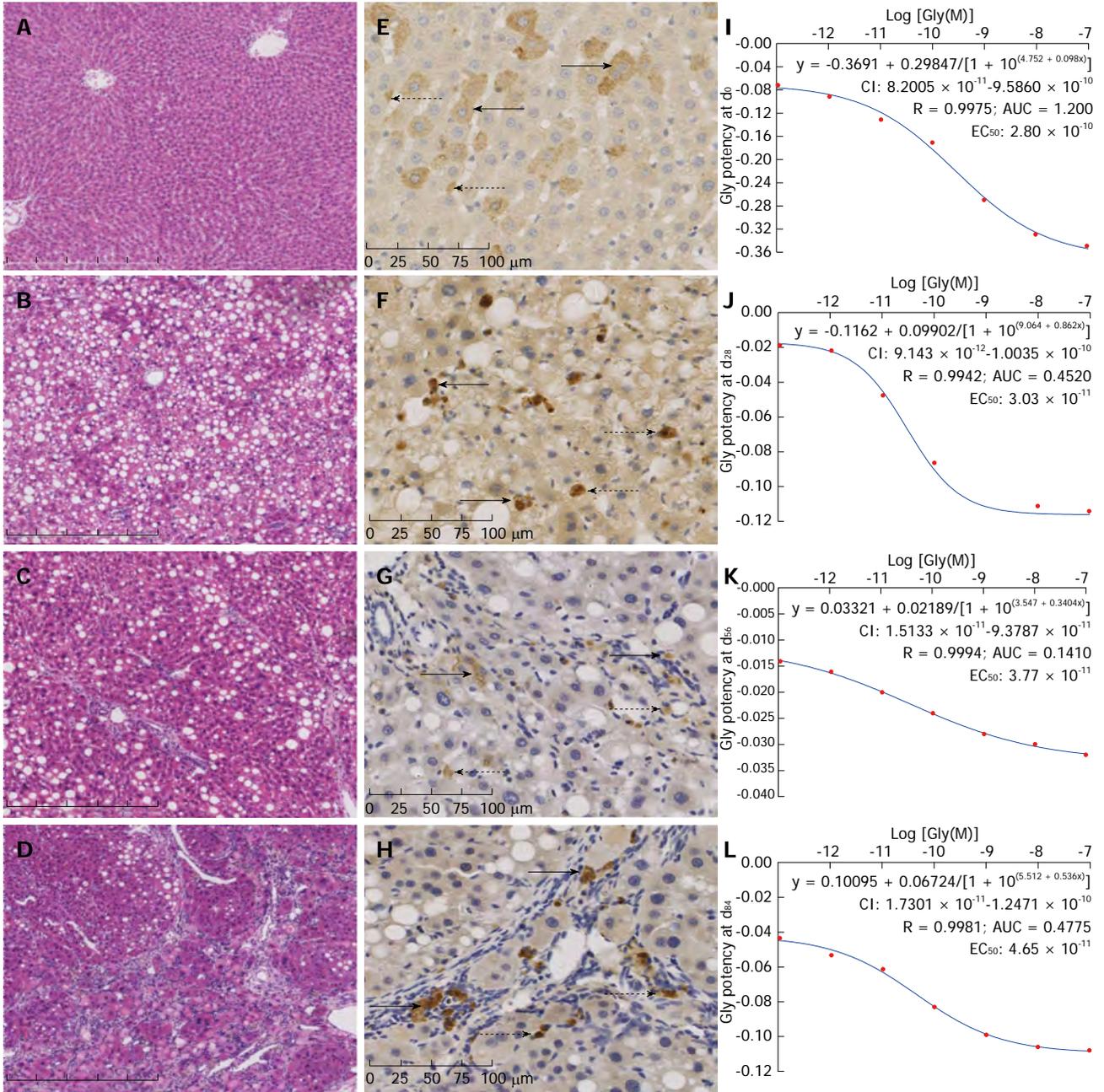
**Localization:** At d<sub>0</sub>, iNOS was mainly located in hepatocytes and scattered stellate cells of lobules (Figure 2E). At d<sub>28</sub>, positive granules were thinner in scattered hepatocytes, thicker in the stellate cells in lobules (Figure 2F). At d<sub>56</sub>, granules in hepatocytes were completely quenched; positive granules limited within macrophages in portal triads and stellate cells in lobules (Figure 2G). At d<sub>84</sub>, thick positive granules located in macrophages in portal triads and stellate cells in pseudo lobules (Figure 2H).

**Quantification:** Levels of existed iNOS per volume in portal triads at d<sub>28</sub>, d<sub>56</sub> and d<sub>84</sub> were significantly increased by 19.39%, -20.00%, and 43.03% from that at d<sub>0</sub> (P < 0.01, Table 3); these at d<sub>56</sub> and d<sub>84</sub> were decreased by 32.99% and increased by 19.80% from that at d<sub>28</sub>, respectively (P < 0.01). So did it increased by 78.79% at d<sub>84</sub> from that at d<sub>56</sub> (P < 0.01).

**Gly reducing PHT**

**Dose-effective relation:** Gly had the same shape of dose-effective curves at d<sub>0</sub> (Figure 2I), d<sub>28</sub> (Figure 2J), d<sub>56</sub> (Figure 2K), d<sub>84</sub> (Figure 2L), respectively, in PHT development. The equations of effective potency to reduce PHT were  $y = -0.3691 + 0.29847/[1 + 10^{(4.75212 + 0.09841x)}]$  (R = 0.9975, P < 0.01),  $y = -0.1162 + 0.09902/[1 + 10^{(9.064 + 0.8616x)}]$  (R = 0.9942, P < 0.01),  $y = -0.03321 + 0.02189/[1 + 10^{(3.547 + 0.3404x)}]$  (R = 0.9994, P < 0.01), and  $y = 0.10095 + 0.06724/[1 + 10^{(5.5121 + 0.5336x)}]$  (R = 0.9981, P < 0.01); EC<sub>50</sub> values with their 95% CIs were  $2.80 \times 10^{-10}$  ( $8.20 \times 10^{-11} - 9.59 \times 10^{-10}$ ),  $3.03 \times 10^{-11}$  ( $9.14 \times 10^{-12} - 1.00 \times 10^{-10}$ ),  $3.77 \times 10^{-11}$  ( $1.51 \times 10^{-11} - 9.38 \times 10^{-11}$ ), and  $4.65 \times 10^{-11}$  ( $1.73 \times 10^{-11} - 1.25 \times 10^{-10}$ ) mol/L, respectively.

**Time-effective relation:** Pathological development af-



**Figure 2** Glycyrrhizinate reduced portal hypertension in isolated portal perfuse rat liver with chronic hepatitis. A: Normal hepatic structure at d<sub>0</sub> (× 100); B: Hepatic degeneration at d<sub>28</sub> (× 100); C: Hepatic fibrosis at d<sub>56</sub> (× 100); D: Hepatic cirrhosis at d<sub>84</sub> (× 100); E: Inducible nitric oxide synthase (iNOS) were located at the hepatocyte (solid arrow) and scattered stellates (dashed arrow) at d<sub>0</sub> (× 400); F: iNOS were located at hepatocytes (solid arrow) and stellates (dashed arrow) in the lobules at d<sub>28</sub> (× 400); G: iNOS were located at stellates (dashed arrow) in the lobules and macrophages (solid arrow) outside of lobules at d<sub>56</sub> (× 400); H: iNOS were located at macrophages (solid arrow) outside of lobules and stellates (dashed arrow) at d<sub>84</sub> (× 400); I: Diammonium glycyrrhizinate (Gly) reducing portal hypertension at d<sub>0</sub>; J: Gly reducing portal hypertension at d<sub>28</sub>; K: Gly reducing portal hypertension at d<sub>56</sub>; L: Gly reducing portal hypertension at d<sub>84</sub>.

ected on Gly potency to reduce PHT. Therefore, EC<sub>50</sub> values of Gly ( $Y \times 10^{-11}$  mol/L) related linearly with durations of PHT ( $y = 0.0289x + 2.1967$ ,  $R = 0.9985$ ,  $P < 0.05$ ).

**Mechanic connection:** Existed iNOS was involved in Gly potency to reduce PHT. Therefore, AUC values of Gly regressed linearly with existed iNOS levels in portal triads at d<sub>28</sub>, d<sub>56</sub>, d<sub>84</sub> in PHT development ( $y = 0.2669x + 0.0931$ ,  $R = 0.9517$ ,  $P < 0.05$ ).

## DISCUSSION

PHT is a common complication in patients with advanced chronic hepatitis<sup>[1]</sup>. It is possible to develop therapeutic candidates against reversible mechanisms in PHT pathogenesis<sup>[1,2]</sup>. In this study, we use PHT model in IPPRL with CCl<sub>4</sub>-induced chronic hepatitis to investigate the potential effects of Gly on PHT, and to explore further the possible underlying mechanisms. NO does directly decrease PHT<sup>[2,18]</sup>. However, eNOS-derived NO

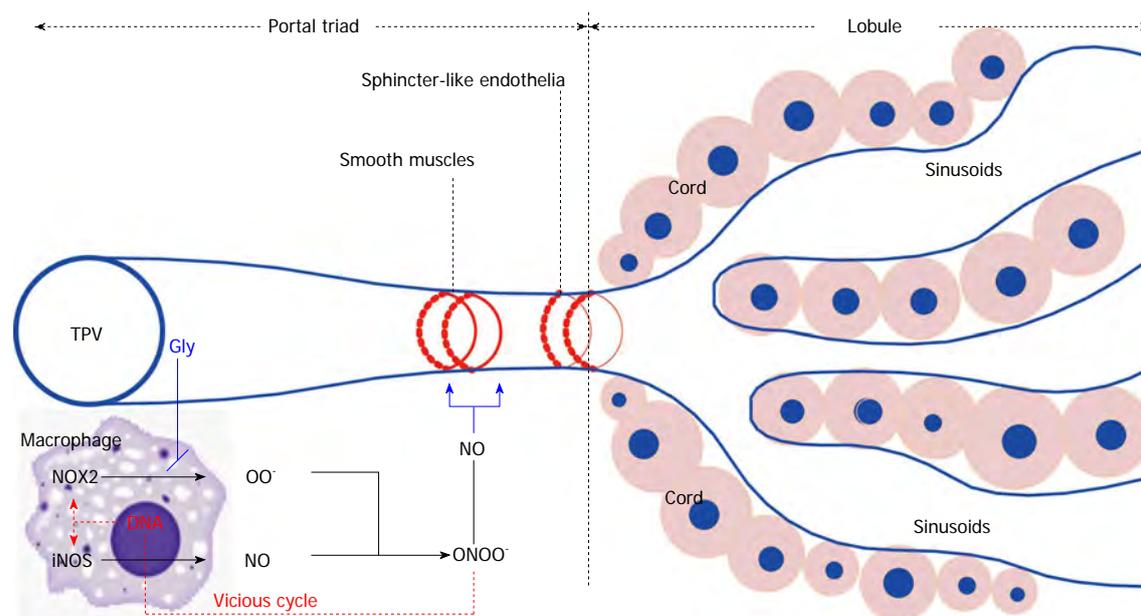


Figure 3 Glycyrrhizinate indirectly reduced portal hypertension. Gly: Diammonium glycyrrhizinate; TPV: Terminal portal venule; NOX: Nicotinamide adenine dinucleotide oxidase; OO·: Superoxide; iNOS: Inducible nitric oxide synthase; NO: Nitric oxide; ONOO·: Peroxynitrite; Gly: Glycyrrhizinate.

precedes systemic hyperemia<sup>[19]</sup>, and iNOS-derived NO reduces itself bioavailability *via* peroxynitrite<sup>[4]</sup>. Extracellular superoxide dismutase in cirrhosis inhibits peroxynitrite generation, increases NO bioavailability, and reduces PHT degree<sup>[3]</sup>. In this regard, local NO bioavailability could be drug targets for PHT. Intrahepatic resistance is mainly originated from terminal portal venules<sup>[5]</sup>. Macrophage-derived iNOS in portal triads contributed to more this resistance. Gly is a molecular from a medical plant<sup>[6]</sup> used for treating PHT<sup>[7-12]</sup>, it can increase NO bioavailability<sup>[12,16]</sup>.

Three findings were obtained in this study. (1) A rat model of PHT with chronic hepatitis was confirmed by ascites, portal pressure, splenic index, serum biomarkers and pathological changes. Four stages of CCl<sub>4</sub>-induced chronic hepatitis were hepatic degenerations, fibrosis, cirrhosis only, cirrhosis with PHT<sup>[13,14,17]</sup>. CCl<sub>4</sub> is transformed into trichloromethyl *via* cytochrome P450 2E1<sup>[17,20]</sup>, trichloromethyl initiated lipid peroxidation consequently. Therefore, free radicals injury is the major mechanism involved in CCl<sub>4</sub>-induced chronic hepatitis, which is associated with the pathogenesis of human disease; (2) As a drug candidate for PHT, Gly had the similar S shapes of dose-effective curves to reduce PHT in IPPRLs with chronic hepatitis at various stages. EC<sub>50</sub> value of Gly at d<sub>0</sub> was nearly 10 times higher than these at d<sub>28</sub>, d<sub>56</sub>, and d<sub>84</sub>, indicating that Gly may be more effective on PHT than on physiological portal pressure. EC<sub>50</sub> values of Gly were geometrically increased at d<sub>28</sub>, d<sub>56</sub>, and d<sub>84</sub>, suggesting that the portal responsibility in pathological status may be gradually decreased along with PHT development; and (3) Existed iNOS disappeared gradually in lobules, and strengthened continuously in portal triads in PHT development. AUC values of Gly were positively correlated

with levels of existed iNOS in portal triads.

Gly had been shown to cause renal and central systemic hypertension *via* inhibiting type 2 11β-hydroxysteroid dehydrogenase<sup>[21]</sup>. Possible mechanisms of Gly to relax directly portal vein included inhibiting gap junction intercellular communications<sup>[22]</sup> and activating peroxisome proliferator-activated receptor<sup>[23]</sup>. As hepatic artery being ligated in this study, IPPRL was made to evaluate the effects of Gly on portal venules alone. Under this condition, portal resistance is mainly originated from both of smooth muscle cells in terminal portal venules and sphincter-like endothelia at hepatic sinusoid inlets<sup>[24]</sup>. Different from extrahepatic vasodilators<sup>[25]</sup>, Gly relaxes indirectly portal vein *via* improving intrahepatic NO bioavailability<sup>[26]</sup>. In this study, AUC values of Gly depend on macrophage-derived iNOS in portal triads. Macrophages infiltrated in portal triads expressed more iNOS and oxidative enzymes, consequently generated more peroxynitrite to decrease NO bioavailability<sup>[15]</sup>. Gly reduces superoxide<sup>[27]</sup>, decreases peroxynitrite, increases NO bioavailability<sup>[4]</sup>, and relieves PHT (Figure 3)<sup>[24]</sup>.

Our results suggested that Gly reduces PHT, which might explain therapeutic effectiveness of Gly-contained prescriptions for patients with PHT ascites. It is also a clue to find more effective candidates related to macrophage-generated iNOS or NADPH oxidase, which partially contributes to the effects of Gly to reduce PHT. Gly or its more effective derivatives should be exploited as candidates to maintain NO bioavailability in terminal portal venules, especially against free radical injury.

## COMMENTS

### Background

Portal hypertension (PHT) is a common complication of chronic hepatitis, with

significant morbidity and mortality in clinic. It is important to develop new drugs for this disease. A sensitive pharmacological model has been established for PHT in the isolated perfused rat livers, which supplies suitable methods for evaluating candidates for PHT.

### Research frontiers

Diammonium glycyrrhizinate (Gly) is one of the representative candidates for PHT in experiment and clinic. It relaxes portal veins in isolated portal perfused rat livers at physiological status. It is also believed that the inducible nitric oxide synthase (iNOS) expressed from infiltrated macrophages in portal triads is important to increase portal resistance. The sensitive pharmacological model was used here to investigate the effect of Gly on PHT, and to exploit further the possible mechanisms of its actions.

### Innovations and breakthroughs

The intrahepatic portal resistance of PHT originates mainly from the smooth muscle cells in terminal portal venules and the sphincter-like endothelia at hepatic sinusoid inlets. Gly is effective for reducing PHT in isolated portal perfused rat livers with chronic hepatitis, with the similar S shape and different potency from its dose-effective curves. As PHT advanced, it was found that more macrophages infiltrated in portal triads and expressed more iNOS. Therefore, macrophage iNOS in portal triads involves in the pathogenesis, on which with Gly acted. The mechanisms of Gly for PHT in isolated portal perfused rat liver with chronic hepatitis are related to increase of nitric oxide (NO) bioavailability.

### Applications

Gly reducing PHT might explain the actions of medical plants in Chinese prescriptions. More effective derivate might be generated from Gly molecule structure. NO bioavailability is a candidate target for PHT.

### Terminology

Peroxyntirite is the anion with the formula ONOO<sup>-</sup>, an unstable structural isomer of nitrate. It derives from both of NO and O<sub>2</sub> in activated macrophages at portal triads with chronic hepatitis. It damages all biomolecules in cells, such as DNA and proteins. Peroxyntirite could let NO availability decrease severely.

### Peer review

The authors investigated the effect of Gly on PHT. They found that Gly reduce PHT in isolated portal perfuse rat livers with chronic hepatitis. Such action may explain Chinese medical herbs for PHT. It also suggests that NO availability involve in the pathogenesis of PHT.

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## Up-to-seven criteria for hepatocellular carcinoma liver transplantation: A single center analysis

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Author contributions: Wang WT proposed the study and was the guarantor; Wang WT and Lei JY performed research and wrote the first draft; Lei JY collected and analyzed the data; all authors contributed to the design and interpretation of the study and to further drafts.

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### Abstract

**AIM:** To detect whether the up-to-seven should be used as inclusion criteria for liver transplantation for hepatocellular carcinoma.

**METHODS:** Between April 2002 and July 2008, 220 hepatocellular carcinoma (HCC) patients who were diagnosed with HCC and underwent liver transplantation (LT) at our liver transplantation center were included. These patients were divided into three groups according to the characteristics of their tumors (tumor diameter, tumor number): the Milan criteria group (Group 1), the in up-to-seven group (Group 2) and the out up-to-seven group (Group 3). Then, we compared long-term survival and tumor recurrence of these three groups.

**RESULTS:** The baseline characteristics of transplant recipients were comparable among these three groups, except for the type of liver graft (deceased donor liver transplant or live donor liver transplantation). There were also no significant differences in the pre-operative  $\alpha$ -fetoprotein level. The 1-, 3-, and 5-year overall survival and tumor-free survival rate for the Milan criteria

group were 94.8%, 91.4%, 89.7% and 91.4%, 86.2%, and 86.2% respectively; in the up-to-seven criteria group, these rates were 87.8%, 77.8%, and 76.6% and 85.6%, 75.6%, and 75.6% respectively ( $P < 0.05$ ). However, the advanced HCC patients' (in the group out of up-to-seven criteria) overall and tumor-free survival rates were much lower, at 75%, 53.3%, and 50% and 65.8%, 42.5%, and 41.7%, respectively ( $P < 0.01$ ).

**CONCLUSION:** Considering that patients in the up-to-seven criteria group exhibited a considerable but lower survival rate compared with the Milan criteria group, the up-to-seven criteria should be used carefully and selectively.

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**Key words:** Up-to-seven criteria; Liver transplantation; Outcome; Hepatocellular carcinoma; Recurrence

**Core tip:** The up-to-seven criteria were introduced several years ago, but there is still no consensus about their effectiveness. Two hundred and twenty patients were divided into three groups according to the characteristics of their tumors: the 1-, 3-, and 5-year overall survival and tumor-free survival rate for the Milan criteria group were higher than that in the up-to-seven criteria group. However, the advanced hepatocellular carcinoma patients' overall and tumor-free survival rates were much lower. So considering that patients in the up-to-seven criteria group exhibited a considerable but lower survival rate compared with the Milan criteria group, the up-to-seven criteria should be used carefully and selectively.

Lei JY, Wang WT, Yan LN. Up-to-seven criteria for hepatocellular carcinoma liver transplantation: A single center analysis. *World J Gastroenterol* 2013; 19(36): 6077-6083 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6077.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6077>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide and is the sixth most common cancer and the third most common cause of cancer death<sup>[1]</sup>. This disease is especially problematic for Asian countries, which have a high prevalence of hepatitis B virus (HBV) and hepatitis C virus infection<sup>[2]</sup>. Effective management of early HCC includes resection, radiofrequency ablation and liver transplantation (LT). Liver transplantation remains the best treatment for small HCC resulting from chronic liver disease, as it both removes the neoplastic lesion and eliminates the underlying disease in a single procedure. However, post-operative recurrence is still a major problem related to HCC LT. Stringent inclusion criteria have been adopted to ensure tumor free survival after LT. The first criteria for HCC LT were introduced by Mazzaferro *et al.*<sup>[3]</sup> and were named the Milan criteria: a solitary lesion of < 5 cm, or 2 to 3 nodules all < 3 cm and without microscopic vascular invasion or extrahepatic disease. Due to the favorable results that have been achieved, *i.e.*, a 5-year post-transplant survival exceeding 70% and a rate of tumor recurrence below 15%, the Milan criteria have been used as the standard selection criteria for HCC LT all over the world<sup>[4,5]</sup>. Several years later, based on greater experience, some groups argued that the Milan criteria should be expanded, as a substantial number of patients with HCC exceeding these criteria could also greatly benefit from transplantation<sup>[6-10]</sup>. The most representative set of new criteria were the University of California San Francisco criteria proposed by Yao *et al.*<sup>[6]</sup>: 1 lesion  $\leq$  6.5 cm in diameter or 2 to 3 lesions, each  $\leq$  4.5 cm in diameter, with a total diameter of  $\leq$  8 cm. Several groups argued the Milan criteria were too strict and excluded some HCC patients from LT, despite the possibility of benefit, and that the criteria should be expanded. Therefore, the Milan group (Mazzaferro *et al.*<sup>[11]</sup>) attempted to expand the Milan criteria and create a new set called the up-to-seven criteria (new Milan criteria): hepatocellular carcinomas with seven as the sum of the size of the largest tumor (in cm) and the number of tumors. In Milan group's study, the up-to-seven groups achieved a 5-year an overall survival rate of 71.2%. Following this study, several other studies demonstrated that the up-to-seven criteria could be useful as a model for evaluating potential candidates for liver transplantation to treat HCC<sup>[12-15]</sup>. Although the up-to-seven criteria have been analyzed all over the world, they have not been as widely accepted as the Milan criteria, even 4 years after their conception. Meanwhile, there is still no research on these criteria in China, where most HBC infections and nearly 55% of worldwide HCC occurs<sup>[16]</sup>. Therefore, in our study, we compared the outcomes of Milan criteria patients with those of up-to-seven criteria patients, and then we evaluated the effectiveness of the up-to-seven criteria as inclusion criteria for HCC LT.

## MATERIALS AND METHODS

From April 2002 to July 2008, 220 HCC patients underwent LT in our liver transplantation center and were included in

our study. All of these patients were diagnosed with HCC based on pre-operative imaging studies, and the diagnoses were confirmed by pathology. Patients with cholangio-hepatocellular cancer or other liver diseases were excluded from this study. All of the tumor characteristics were evaluated by histological examination. Of these 220 cases, 58 patients met the Milan criteria (Group 1), 90 patients met the up-to-seven criteria (Group 2) and 130 patients did not meet either the Milan criteria or up-to-seven criteria (Group 3). We retrospectively collected the data of these three groups and then compared their baseline characteristics, intraoperative data, post-operative recovery and long-term survival, including the overall survival, tumor-free survival and recurrence rate. All of the data were collected from the Chinese Liver Transplant Registry (<http://www.cltr.org>).

In our study, the grafts for liver transplantation were from living right lobe donors and deceased donors. No prisoners were included as donors, and all of the whole liver grafts were donations after cardiac death. All of these donations were volunteered by the donor or the family. For grafts that came from living donors, the donor was required to be within three degrees of consanguinity with the recipient, as verified by a DNA test, and all of the living donor liver transplantations were performed after obtaining approval from the Ethics Committee of the West China Hospital and local authorities. All of the donations were voluntary and altruistic. We informed the donors and their families of the possible risks of donor hepatectomy. Written consent was provided by the donors for the storage of their information in the hospital database and its use for research.

The surgical procedures performed on the donor and the recipients are described in our previous reports<sup>[17-19]</sup>. Routine post-LT triple-immunosuppressive treatment in our center includes tacrolimus or cyclosporin, mycophenolate mofetil, and steroids. For patients with HBV infection, the anti-HBV protocol after LT included lamivudine combined with low-dose hepatitis B immunoglobulin therapy<sup>[20]</sup>. The doses of tacrolimus and cyclosporine were adjusted based on the measured serum level.

### Statistical analysis

All of the data were managed and analyzed using SPSS 17.0 statistical software. Descriptive variables such as age, MELD score, and tumor diameter were expressed as the mean  $\pm$  SE. Categorical data such as gender and graft type were computed using the Pearson  $\chi^2$  test or Fisher's exact test. The overall survival and tumor-free survival rates were calculated and compared using Kaplan-Meier analysis. Only tumor-related deaths were included in the recurrence-free survival analysis. The log-rank test was performed to compare survival curves. Two-sided *P* values were computed and a difference of *P* < 0.05 was adopted as the threshold for statistical significance.

## RESULTS

### Baseline characteristics

Over six years, 220 HCC patients underwent LT at our

**Table 1** Baseline, tumor characteristics, and overall and tumor-free survival rates of the liver transplantation recipients

	Milan criteria (Group 1), n = 58	In up-to-seven criteria (Group 2), n = 90	Out up-to-seven criteria (Group 3) n = 120	P value 1 vs 2	P value 2 vs 3	P value 1 vs 3
Baseline characteristics						
Gender (M/F)	51/7	81/9	109/11	0.859	0.648	0.552
Age (yr)	48.4 ± 10.9	46.8 ± 10.8	45.4 ± 10.0	0.390	0.315	0.067
BMI (kg/m <sup>2</sup> )	23.3 ± 2.4	23.5 ± 2.3	23.3 ± 2.0	0.715	0.739	0.963
Underlying LD				0.775	0.452	0.312
HBV	54	83	78			
HCV	0	1	1			
No hepatic virus	4	6	16			
HBV-DNA(-/+)	29/39	45/45	82/38	0.838	0.070	0.174
DDLT/LDLT	36/22	62/28	98/23	0.393	0.022	0.003
Child score (A/B/C)	29/16/13	49/23/18	66/37/17	0.492	0.671	0.259
Meld score	12.6 ± 6.3	11.8 ± 6.3	10.8 ± 5.2	0.430	0.242	0.051
Donor Characteristics						
Donor age (yr)	37.2 ± 10.2	37.4 ± 11.4	36.9 ± 9.8	0.892	0.558	0.782
Donor BMI (kg/m <sup>2</sup> )	22.6 ± 3.1	22.7 ± 3.5	23.5 ± 3.6	0.921	0.672	0.691
Donor risk index	1.44	1.41	1.49	0.721	0.322	0.675
Tumor characteristics						
Total diameter (cm)	4.1 ± 1.5	4.4 ± 1.8	11.7 ± 4.4	0.307	0.000	0.000
Number (1/2/3/4-5/diffuse)	43/13/2/0/0	54/18/3/15/0	49/17/11/9/34	0.024	0.000	0.000
AFP level (ng/mL)	1361.7 ± 4633.1	1122.4 ± 3764.5	2320.6 ± 10672.4	0.735	0.316	0.520
Overall survival rate				0.036	0.001	0.000
1 yr	94.80%	87.80%	75%			
3 yr	91.40%	77.80%	53.30%			
5 yr	89.70%	76.60%	50%			
Tumor-free survival rate				0.046	0.000	0.000
1 yr	91.40%	85.60%	65.80%			
3 yr	86.20%	75.60%	42.50%			
5 yr	86.20%	75.60%	41.70%			

BMI: Body mass index; LD: Liver disease; HBV-DNA: Hepatitis B virus DNA; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation; AFP:  $\alpha$ -fetoprotein; HCV: Hepatitis C virus.

transplantation center, and all of them were followed up for at least 5 years. The baseline characteristics of the donors and recipients are summarized in Table 1. There were no significant differences among three groups with respect to recipient gender, age, or body mass index (BMI). The most common etiology of cirrhosis was hepatitis B infection. There were only 2 cases of hepatitis C infection. No differences were observed in either underlying liver disease (hepatitis virus) or HBV-DNA level. However, fewer patients underwent LDLT in the out up-to-seven group: 19.2% (23 cases) of patients in the out up-to-seven group, 37.9% (22 cases) patients in the Milan group, and 31.3% (28 cases) patients in the in up-to-seven criteria group ( $P < 0.05$ ) underwent LDLT. The pre-LT liver function, determined by the Meld score and Child score, were also not different among the three groups. There were also no differences among the three groups with respect to donor characteristics, including donor age, BMI and donor risk index.

### Tumor characteristics

There was no difference in the total diameter of the tumors between the Milan criteria and the up-to-seven criteria groups ( $P = 0.307$ ). However, the diameter in the Out up-to-seven criteria group was much larger than that in the other two groups ( $P = 0.000$ ). The out up-to-seven criteria group had the highest tumor number, followed

by the up-to-seven criteria group. There were no diffused targets in either the Milan group or the up-to-seven criteria group. However, there were 34 cases with diffused targets in the out up-to-seven criteria group. Seventy-on cases were diagnosed with macrovascular invasion by pre-LT imaging scans, and the diagnoses were confirmed by histological examination. However, there was no significant difference in the AFP level among the three groups ( $P > 0.05$ , shown in Table 1). One new target was found in the explanted liver of a patient in the Milan group, and 3 new targets were found in the up-to-seven group. The diameters of these new targets ranged from 0.8 to 3.0 cm.

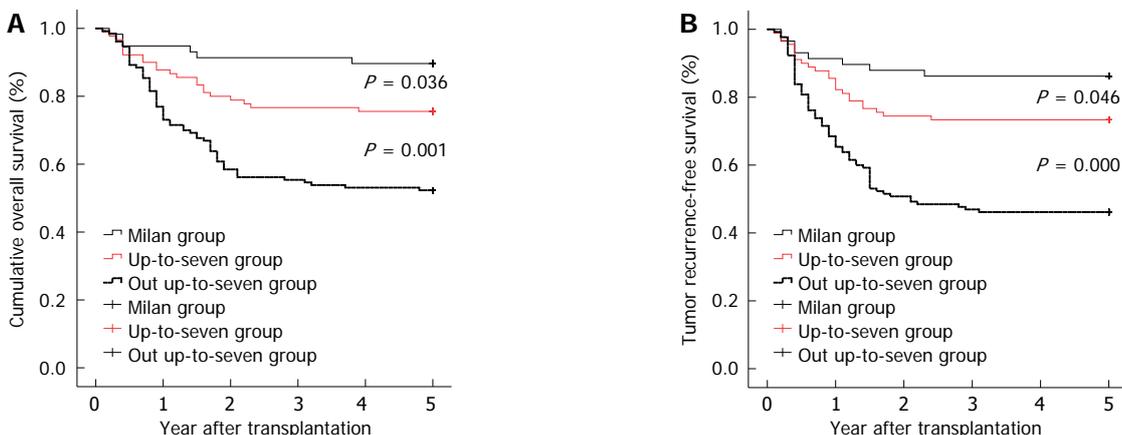
### Survival and tumor recurrence

The length of follow-up for all the patients in our study was at least 5 years, and no significant differences were observed among the groups. The 1-, 3-, and 5-year overall and tumor-free survival rates of Milan criteria group were superior to those of the up-to-seven patients [94.8% vs 87.8%, 91.4% vs 77.8%, and 89.7% vs 76.6%, respectively ( $P = 0.036$ ) and 91.4% vs 85.6%, 86.2% vs 75.6%, and 86.2% vs 75.6%, respectively ( $P = 0.046$ )]. The 1-, 3-, and 5-year overall survival rates (75%, 53.3%, and 50%, respectively) and tumor-free survival rates (65.8%, 42.5%, and 41.7%, respectively) in the patients whose HCCs did not meet the up-to-seven criteria were much

**Table 2 Site of hepatocellular carcinoma recurrence or metastasis after liver transplantation**

	Liver	Lung	Bone	Liver + lung	Lung + bone	Intra-abdominal	Other
Milan group	3	1	0	1	0	0	0
In up-to-seven group	8	3	1	4	0	0	0
Out up-to-seven group	16	21	1	9	10	4	3

Other: Lung and brain (1 case), lung and spine liver, lung and bone.



**Figure 1 Survival and tumor recurrence.** A: Overall survival rates of two groups ( $P = 0.498$ ); B: Tumor recurrence-free rates of two groups ( $P = 0.838$ ).

lower than those of the other patients ( $P < 0.01$ , Table 1). The most common reason for mortality within 1 year was complications, and not tumor recurrence, for the Milan criteria group and up-to-seven criteria group (none of the patients in the Milan criteria group and 2 patients (18.2%) in the up-to-seven group died from tumor recurrence within 1 year). However, most of the 26 (86.7%) deaths within 1 year in the out up-to-seven criteria group were due to tumor recurrence. However, 1 year after LT, the most common cause of mortality for all three groups was tumor recurrence.

The most common site of recurrence was the liver graft for all three groups, and lung metastasis was the second common site of recurrence. Additionally, some patients were diagnosed with combined organ recurrence or metastasis to the liver, lung or bone (Table 2). Although more patients were diagnosed with recurrence or metastasis in the out up-to-seven group than in the other groups, the site of recurrence or metastasis was not significantly different among the three groups. In the 71 cases who were diagnosed with macrovascular invasion, 54 (71.6%) tumors recurred or metastasized after LT (Figure 1).

## DISCUSSION

All of the baseline characteristics were comparable among the three groups, except for the source of the liver graft, as there were many fewer living donor liver transplantations in the up-to-seven group compared with the Milan criteria group. The main reason for this selective bias was the absence of a national organ allocation

system such as the United Network for Organ Sharing in China; the lack of such a system means that the criteria for HCC living donor liver transplantation are much stricter than deceased donor liver transplantation because living donor liver graft harvest involves potential risks to donors, including death<sup>[18,21]</sup>. However, this bias was assumed to have no impact on our analyses, as the results of another of our studies indicated that there were no significant differences in postoperative complications, tumor recurrence rate, survival rate, and HBV recurrence between deceased donor liver transplant (DDLT) and live donor liver transplantation (LDLT) patients<sup>[22]</sup>. Many other reports<sup>[23,24]</sup> have also indicated that there is no difference in long-term outcomes between DDLT and LDLT. Some published papers<sup>[25]</sup> have even indicated that early graft regeneration and features specific to living-donor liver transplantation (LDLT) may adversely influence the recurrence of HCC. Our data indicated that LDLT was performed more frequently in the Milan criteria group, so this selective bias did not affect the long-term survival rate.

Since the Milan criteria for HCC LT were proposed in 1996, dozens of models from all over the world have been developed to expand the indications for LT for patients with HCC without compromising overall or tumor-free survival compared with patients who underwent LT based on the Milan criteria. The main aim of expanding the Milan criteria was to include more HCC patients but maintain comparable outcomes. Although few reports have suggested that tumor involvement in the portal branches is a contraindication for LT<sup>[26,27]</sup>, there is general agreement among various researchers

that patients presenting macrovascular invasion or extrahepatic spread should be excluded from LT given the unacceptable rate of recurrence<sup>[10]</sup>; this presumption was confirmed by our analysis, which showed a very high recurrence rate (71.6%) in these cases. However, the proposed expanded criteria appear to be vague: the upper diameter of a single tumor ranges from 5 to 9 cm<sup>[6,28-30]</sup>, and the highest number of tumors ranges from 3 to unlimited<sup>[6,28,29,31]</sup>. Some published studies did not even impose upper limits on the tumor number or diameter<sup>[32,33]</sup>. Thirteen years after the Milan criteria were developed, the Mazzaferro group proposed an expanded set of criteria called the up-to-seven criteria (new Milan criteria). In their study, the 5-year overall survival rate for Milan group patients was 73.3%; this finding is comparable to our results, which showed a 5-year overall survival of 75%. However, for the up-to-seven criteria patients, the overall survival rate in our study was 53.3%, which was much lower than the 71.2% reported in the Mazzaferro *et al*<sup>[11]</sup>. Several studies have evaluated the effectiveness of using the up-to-seven criteria as inclusion criteria for HCC LT<sup>[14,15]</sup>. de Ataide *et al*<sup>[12]</sup> directly compared the long-term outcomes of a Milan criteria group and an up-to-seven criteria group. Their results showed that the post liver transplantation survival rates were 87.7%, 74.5% and 65.3% at 1, 3, and 5 years among patients who met the up-to-seven criteria, and these rates were similar to those in patients meeting the Milan criteria. However, there is still some disagreement regarding the up-to-seven criteria. In a letter to Mazzaferro and his colleagues, Sotiropoulos *et al*<sup>[13]</sup> stated that although the up-to-seven criteria are based on objective tumor characteristics such as tumor size, tumor number, and microvascular invasion, these characteristics represent pathology findings and not preoperative objective tumor characteristics, and therefore, the up-to-seven criteria are illusive and not applicable in clinical practice. In the present study, 71 patients (59.1%) in the out up-to-seven criteria group showed macrovascular invasion, which was an independent risk factor for HCC recurrence after LT. Our data on the tumor characteristics for this analysis all come from pre-operative imaging data and were confirmed by the histological examination. Only a few targets (4 cases) were found in the explanted liver. We did not evaluate the new targets in the out up-to-seven group because there were some cases with diffused tumors, so finding and calculating new tumor targets would have been very difficult in these patients.

Our Milan criteria patients exhibited a 89.7% 5-year overall survival rate, and this rate is higher than that in some reports<sup>[10]</sup> but comparable with those in many other reports<sup>[29,34,35]</sup>. Although the up-to-seven criteria group included 90 patients, which was much higher than the number of patients in the Milan criteria group (53 cases), the main aim of expanding the Milan criteria was to include more HCC patients without compromising outcomes; this, in our study, long-term (5-year) survival was much lower in the up-to-seven group. The concept of the

“metro ticket” has been used to demonstrate this point, that is, expanding the criteria to allow both increased size and increased number of nodules resulted in an increased risk of recurrence. The further the criteria are expanded, the higher the risk in terms of survival<sup>[4]</sup>. However, although the survival rate in the up-to-seven criteria group was lower than that in the Milan criteria group, the 5-year overall survival rate was still considerable at 76.6%, which was comparable with the Milan criteria 5-year survival rate in many other reports<sup>[10]</sup>. Meanwhile, the overall survival rate of patients who met the up-to-seven criteria was much higher than those who did not meet up-to-seven criteria (5-year survival rate: 76.6% *vs* 50%). These comparisons suggest that the up-to-seven criteria may be accepted.

The limitations of this study include the fact that these data were retrospectively collected and analyzed. A future randomized study would be the best way to evaluate the effectiveness of the up-to-seven criteria as inclusion criteria for HCC LT. However, this ideal design would be very difficult to implement due to logistical challenges. In addition, a large multicenter study comparing a larger number of patients with HCC LT would be ideal for future analyses.

In conclusion, considering the differences in long-term outcome, care should be taken when using the up-to-seven criteria rather than the Milan criteria to include HCC patients in LT.

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## COMMENTS

### Background

Liver transplantation remains the best treatment for small hepatocellular carcinoma (HCC) resulting from chronic liver disease. However, post-operative recurrence is still a major problem related to HCC liver transplantation (LT). Stringent inclusion criteria have been adopted to ensure tumor free survival after LT. The first criteria for HCC LT were introduced in 1996 and were named the Milan criteria. Several years later, based on greater experience, some groups argued that the Milan criteria should be expanded. Therefore, the Milan group attempted to expand the Milan criteria and create a new set called the up-to-seven criteria (new Milan criteria): HCCs with seven as the sum of the size of the largest tumor and the number of tumors.

### Research frontiers

Although the up-to-seven criteria have been analyzed all over the world, they have not been as widely accepted as the Milan criteria, even 4 years after their conception. Meanwhile, there is still no research on these criteria in China, where most hepatitis B virus and hepatitis C virus infections and nearly 55% of worldwide HCC occurs.

### Innovations and breakthroughs

The up-to-seven criteria were introduced several years ago, but there is still no consensus about their effectiveness. Two hundred and twenty patients were divided into three groups according to the characteristics of their tumors in the authors' center. Considering that patients in the up-to-seven criteria group exhibited a considerable but lower survival rate compared with the Milan criteria group, the up-to-seven criteria should be used carefully and selectively.

### Applications

Considering the differences in long-term outcome, care should be taken when

using the up-to-seven criteria rather than the Milan criteria to include HCC patients in LT.

**Peer review**

This is an interesting study comparing liver transplant outcomes in 3 groups of patients with different stage of HCC.

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## Overexpression of insulin-like growth factor- I receptor as a pertinent biomarker for hepatocytes malignant transformation

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### Abstract

**AIM:** To investigate the dynamic features of insulin-like growth factor- I receptor (IGF- I R) expression in rat hepatocarcinogenesis, and the relationship between IGF- I R and hepatocytes malignant transformation at mRNA or protein level.

**METHODS:** Hepatoma models were made by inducing with 2-fluorenylacetylamide (2-FAA) on male Sprague-Dawley rats. Morphological changes of hepatocytes were observed by pathological Hematoxylin and eosin staining, the dynamic expressions of liver and serum IGF- I R were quantitatively analyzed by an enzyme-linked immunosorbent assay. The distribution of hepatic IGF- I R was located by immunohistochemistry. The fragments of *IGF- I R* gene were amplified by reverse transcription-polymerase chain reaction, and confirmed by sequencing.

**RESULTS:** Rat hepatocytes after induced by 2-FAA were changed dynamically from granule-like degeneration, precancerous to hepatoma formation with the progressing increasing of hepatic mRNA or IGF- I R expression. The incidences of liver IGF- I R, IGF- I R mRNA, specific IGF- I R concentration (ng/mg wet liver), and serum IGF- I R level (ng/mL) were 0.0%, 0.0%,  $0.63 \pm 0.17$ , and  $1.33 \pm 0.47$  in the control; 50.0%, 61.1%,  $0.65 \pm 0.2$ , and  $1.51 \pm 0.46$  in the degeneration; 88.9%, 100%,  $0.66 \pm 0.14$ , and  $1.92 \pm 0.29$  in the precancerosis; and 100%, 100%,  $0.96 \pm 0.09$ , and  $2.43 \pm 0.57$  in the cancerous group, respectively. IGF- I R expression in the cancerous group was significantly higher ( $P < 0.01$ ) than that in any of other groups at mRNA or protein level. The closely positive IGF- I R relationship was found between livers and sera ( $r = 0.91$ ,  $t = 14.222$ ,  $P < 0.01$ ), respectively.

**CONCLUSION:** IGF- I R expression may participate in rat hepatocarcinogenesis and its abnormality should be an early marker for hepatocytes malignant transformation.

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**Key words:** Hepatoma; Insulin-like growth factor- I re-

ceptor; Immunohistochemistry; Gene amplification; Sequencing; Rat hepatoma model

**Core tip:** The abnormality of insulin-like growth factor- I receptor (IGF- I R) expression was progressively increased in hepatocarcinogenesis at mRNA or protein level, and there was a positive correlation between circulating blood and liver IGF- I R, suggesting that the over-expression of liver IGF- I R release into blood, and serum IGF- I R should be an early useful marker for monitoring malignant transformation of rat hepatocytes.

Yan XD, Yao M, Wang L, Zhang HJ, Yan MJ, Gu X, Shi Y, Chen J, Dong ZZ, Yao DF. Overexpression of insulin-like growth factor- I receptor as a pertinent biomarker for hepatocytes malignant transformation. *World J Gastroenterol* 2013; 19(36): 6084-6092 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6084.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6084>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death, especially in the inshore area of the Yangtze River<sup>[1-3]</sup>. Multiple pathogenic factors, including infection with hepatitis B virus or hepatitis C virus, chronic hepatitis and cirrhosis with the subsequent multistage pathogenesis, have been extensively studied<sup>[4-6]</sup>. Recent advances in molecular genetics have identified a large number of activated or suppressed genes may play significant roles in HCC<sup>[7-9]</sup>. Insulin-like growth factor (IGF) signaling is specifically required for hepatoma progression<sup>[10]</sup>, especially in IGF- II and IGF- I receptor (IGF- I R) which are the very important signaling contributors. Surgical liver transplantation or resection remains the mainstay of curative therapy for patients in the early stage of HCC<sup>[11,12]</sup>.

Carcinoembryonic IGF- II was reported to be over-expressed at different stages of HCC development<sup>[12]</sup>. Integrative genomic analysis showed enrichment of IGF activation in the proliferation subclass of HCC. Effective blockage of IGF signaling with A12 provides the rationale for testing this therapy in clinical trials<sup>[13]</sup> and confirmed that it plays the biologic role primarily through IGF- I R<sup>[14]</sup>. Although the IGF- I R expression is distributed widely in multiple tissues, it is high in fetal liver but not in adult liver<sup>[10]</sup>. IGF- I R is an important regulatory factor necessary for cell growth and differentiation, provides mitogenic signal for tumor growth<sup>[15]</sup>, exhibits a very potent anti-apoptotic activity<sup>[16]</sup>, induces vascular endothelial growth factor expression, and able to maintain the cell phenotype transformation in carcinogenesis<sup>[17,18]</sup>. However, the features of IGF- I R expression during hepatocytes malignant transformation have not yet been reported. Thus, the objectives of the present study were to investigate the dynamic expression features of IGF- I R

in rat hepatocarcinogenesis, and the relationship between IGF- I R expression and hepatocyte malignant transformation at mRNA or protein level.

## MATERIALS AND METHODS

### *Hepatoma model and pathological analysis*

Rat hepatoma models were made as described previously<sup>[13]</sup>. Briefly, 54 male Sprague-Dawley rats (4-wk-old, body weight 120-130 g) from the Experimental Center of Medical Animals, Nantong University, China were used to induce HCC development by fed foods with 0.05% 2-fluorenylacetylacetamide (2-FAA, Sigma). Rats were monitored daily for survival and weight loss, recorded their clinical signs and sacrificed at different days. All procedures performed on the animals were conducted in accordance with the guidelines for experimental animals approved by the Animal Care and Use Committee of Nantong University, China. According to the plan, 5 mL of blood was drawn from rat hearts and serum was separated for IGF- I R test, and livers were used for pathology, immunohistochemistry, total RNA extraction, and quantitative analysis of IGF- I R expression. Rat liver tissues were fixed in 10% formaldehyde solution, sectioned after dehydration, and paraffin embedding and then performed with HE staining to observe morphological changes under microscope.

### *Total RNA extraction*

Liver tissue (20 mg) was homogenized on ice after adding 1.0 mL of TRIzol reagent in a glass homogenizer which was made RNase-free by 0.1% DEPC water. One milliliter slurry was transferred into the tubes and centrifuged at 15000 rpm for 10 min at 4 °C. 0.8 mL of the supernatant was put into another tube, and then 0.2 mL of chloroform was added into the tube, mixed by vortexing 2 min, centrifuged at 10000 rpm for 10 min at 4 °C, 0.3 mL of the supernatant was collected into another RNase-free tube, equivalent 100% isopropanol and then another 0.5 mL of 50% isopropanol were added, mixed gently for 30 s, centrifuged at 15000 rpm for 5 min at 4 °C. The supernatant was removed, the RNA pellets were washed with 1.0 mL of 75% ethanol, mixed, and centrifuged at 15000 rpm for 5 min at 4 °C. The RNA pellets were air dried 3 min at room temperature, reconstituted in 20 µL of 0.1% lithium chloride, and incubated at 50 °C for 5 min. The purity and concentration of total RNA was estimated, and the ratio was calculated according to absorbance (*A*) readings at *A*<sub>260</sub>/*A*<sub>280</sub> nm.

### *IGF- I R cDNA synthesis*

In a 0.2 mL tube, 2 µg of RNA was combined with 1 µL of random primer (Oligo-dT<sub>18</sub>, 0.5 µg/µL) and adjusted volume to 12 µL with dH<sub>2</sub>O, centrifuged for 5 s. Denature RNA and primer were incubated at 70 °C for 5 min and placed on ice for 30 s, then centrifuged for 5 s, placed on ice and added the reagents as follows: 4 µL of 5 X cDNA Synthesis Buffer, 1 µL of RNase OUT (20 U/µL), 2 µL dNTP Mix (10 mmol/L), centrifuged for 5 s,

incubated at 37 °C for 5 min, placed on ice for 30 s, then added 1 µL of RevertAid M-MuLV Reverse Transcriptase (20 U/µL). Lastly, the reaction was terminated by heating at 70 °C for 5 min after incubating for 60 min at 37 °C.

#### **Primers and nested polymerase chain reaction**

According to rat IGF- I R mRNA full-length sequence (NM052807), and the primers were designed by Premier Primer 5.0 software. External primer P<sub>1</sub>: 5'-CTGCGGCGA TGAAGAAAAGA-3'(nt 1038-1057) and P<sub>2</sub>: 5'-TG-GAGGTGAAACGGAGAAC A-3' (nt 1521-1540), 503 bp; Internal primer P<sub>3</sub>: 5'-ATGCCCTGGTCTCCCTTGTC CT T-3' (nt 1229-1250) and P<sub>4</sub>: 5'-TTTGCTCTGCC-GTCCCTTTGTT-3' (nt 1449-1470), 242 bp. IGF- I R cDNA was amplified by a nested Polymerase chain reaction (PCR), with the 1<sup>st</sup> stage was performed in 2 µL of cDNA (0.1 µg/µL), 2.0 µL of the mix primers (P<sub>1</sub>, P<sub>2</sub>), 12.5 µL of Priemix Taq, and adjusted volume to 25 µL with dH<sub>2</sub>O under the following conditions: 94 °C for 5 min followed by 35 cycles of 94 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min and a final extension step of 10 min at 72 °C; the 2<sup>nd</sup> stage was performed according to above same condition except for 1 µL of the 1<sup>st</sup> PCR solution, and 2 µL of the mix primers (P<sub>3</sub>, P<sub>4</sub>), and finally, the PCR product was stored at 4 °C for analysis.

#### **Electrophoresis and sequencing**

Agarose (0.375 g) was added to 25 mL of TAE buffer, superheated for 3 times. When the agarose cooled to about 50 °C, 1.5 µL of ethidium bromide (10 mg/mL) was added, poured into taped gel tray, placed the combs. 300 mL of TAE buffer was poured into the electrophoresis box. Mixed 8 µL of PCR product with 1 µL of loading dye and put into a well, DNA ladder into another gel well, than electrophoresized on 1.5% agarose gel at 100 V for 30 min, observed with UV Transilluminator at 320 nm, Cut and purified the DNA fragments from the gels for sequencing in MegaBACE DNA sequencer, and the alignment of nucleotide sequences was compared with the original IGF- I R gene sequence.

#### **Immunohistochemistry**

Rat livers were fixed in 10% neutral formalin, embedded in paraffin and 4 µm of section was cut, dewaxed, hydrated, and treated with 3% H<sub>2</sub>O<sub>2</sub> to quench endogenous peroxidase; tissue antigens were retrieved with microwave; and then the sections were blocked by normal animal serum. The first antigen of IGF- I R was dropped on tissue section overnight at 4 °C and thoroughly washed by PBS. Subsequently, the slides were washed in PBS again after adding biotinylated secondary antibody and incubating for 10 min, then added drops of streptomycin avidin-peroxidase (S-P), incubated for 10 min at room temperature, rinsed by dH<sub>2</sub>O and added DAB. After immunostaining, the slides were restrained with hematoxylin, dehydrated in a series of ethanol solutions, covered with neutral gum,

and observed under the microscope (Olympus BX 50). The negative control included 0.01 mol/L PBS instead of the primary and secondary antibodies and S-P agent. Breast-cancerous tissues with positive IGF- I R expression served as positive control. Sensitive gamma-glutamyl-transferase (GGT) is used as a control at the same stage<sup>[19]</sup>. As IGF- I R-positive expression was evident as brown particles in tissues. IGF- I R expression in livers was divided in weakly positive (+, 10%-25% positive cells); moderately positive (++, 26%-75%); and strongly positive (+++, > 75%).

#### **Extraction and measurement of total protein**

Liver tissue (50 mg) was homogenized for 3 min after the addition of 1.0 mL of PBS. The slurry was transferred into tubes (1.5 mL) and centrifuged at 5000 rpm for 4 min at 4 °C. The supernatant was put into another tube and stored at 4 °C. The concentration of total protein was determined by BCA protein measuring kit. Working Reagent was prepared by mixing BCA Reagents (50:1, Reagent A:B), 10 µL of each protein standard was pipetted into a 96-well plate and diluted to 100 µL with 0.9% normal saline. Besides, the standards were added into the 96-well plate as follows: 0, 1, 2, 4, 8, 12 and 20 µL, and each well was added up to 20 µL with diluent. Then 5 µL of tissue proteins were pipetted into the wells and adjusted volume to 20 µL with 0.9% normal saline. In the end, added 200 µL BCA Working Reagent, incubated at 37 °C for 30 min and the absorbance was measured at 562 nm on a plate reader.

#### **Detection of serum and liver IGF- I R levels**

The levels of liver IGF- I R were detected using a rat IGF- I R enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. For the study, 100 µL of standard or tissue protein were separately put into each well of a 96-well ELISA plate, mixed gently for 30 s, incubated for 120 min at 37 °C. 100 µL of biotinylated anti-rat-IGF- I R was added, and incubated for 60 min at 37 °C. And then 100 µL of HRP-conjugate was added to the wells, and incubated for 60 min at 37 °C. Next, 100 µL of enzyme substrate working fluid was added to each well in dark for 5-10 min at 37 °C. Then, 100 µL of stop solution was put into each well, and absorbance was read at 492 nm. During the procedure, washing the plate was according to the ELISA kit. The levels of serum IGF- I R were detected in the same way.

#### **Statistical analysis**

Data were expressed as mean ± SD and subjected to analysis of variance. Differences between groups were assessed by using Newman-Keuls test, the Fisher's exact test or Rank-Sum test. Statistical significance was accepted at the level of *P* value less than 0.05 by using the Stata 7.0 software.

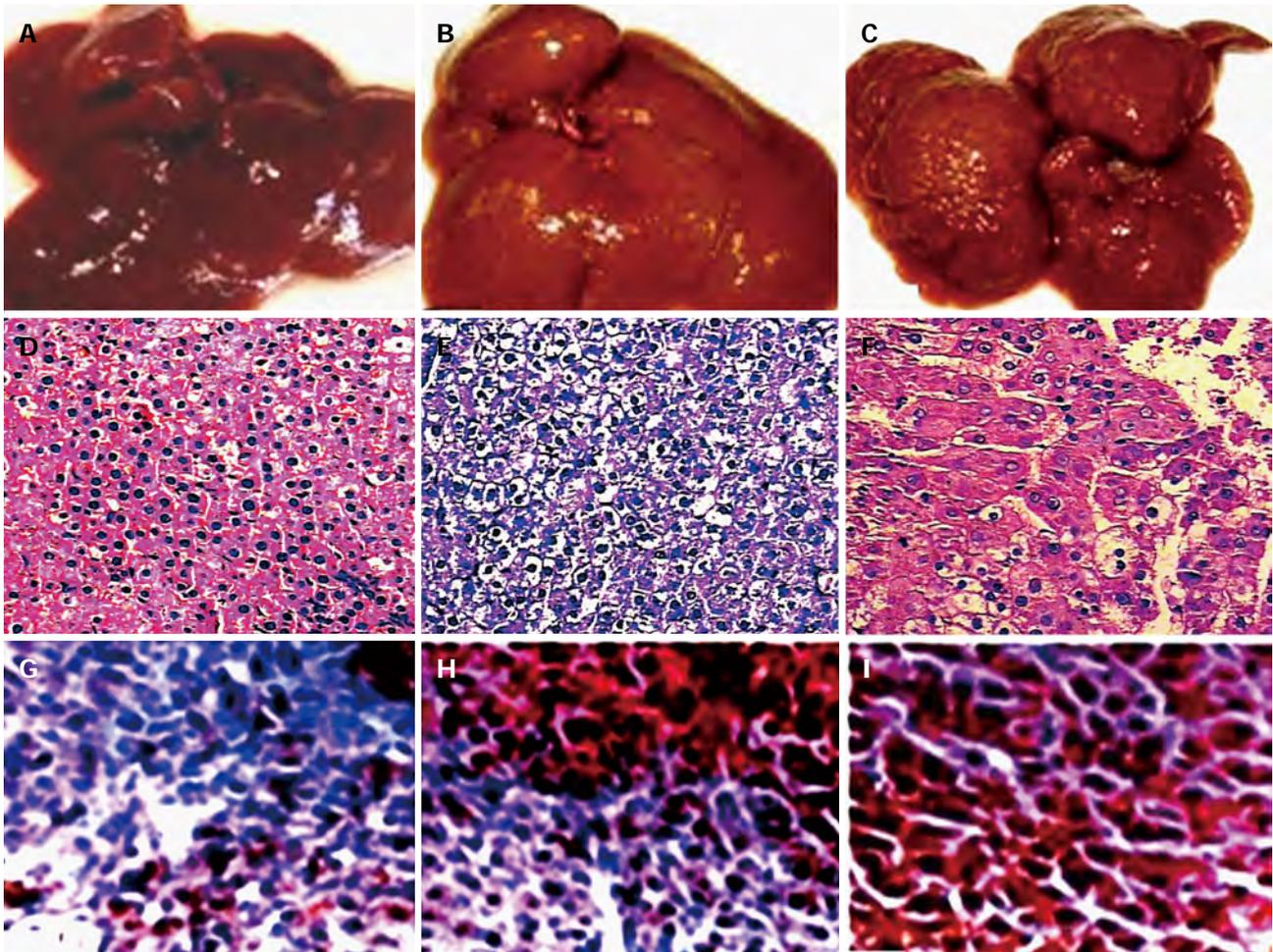


Figure 1 Rat livers and the histopathological alterations of rat tissues during malignant transformation of hepatocytes. A-C: The appearances of rat livers at different stages at early stage (A), at intermediate stage (B), and at later stage (C); D-F: The histopathology alteration (hematoxylin and eosin staining) of the corresponding rat liver section ( $\times 200$ ); G-I: The immunohistochemical confirmation of corresponding rat hepatic gamma-glutamyl transferase at the same stage ( $\times 200$ ).

**Table 1** Histopathological changes of liver in hepatocarcinogenesis (hematoxylin and eosin staining)

Group	n	Histopathological changes in rat liver tissue			
		Control	Degeneration	Precancerosis	HCC
Control	12	12	0	0	0
Experimental					
2 <sup>nd</sup> wk	6	0	6	0	0
4 <sup>th</sup> wk	6	0	6	0	0
6 <sup>th</sup> wk	6	0	5	1	0
8 <sup>th</sup> wk	6	0	1	4	1
10 <sup>th</sup> wk	6	0	0	3	3
12 <sup>th</sup> wk	6	0	0	1	5
Total	48	12	18	9	9

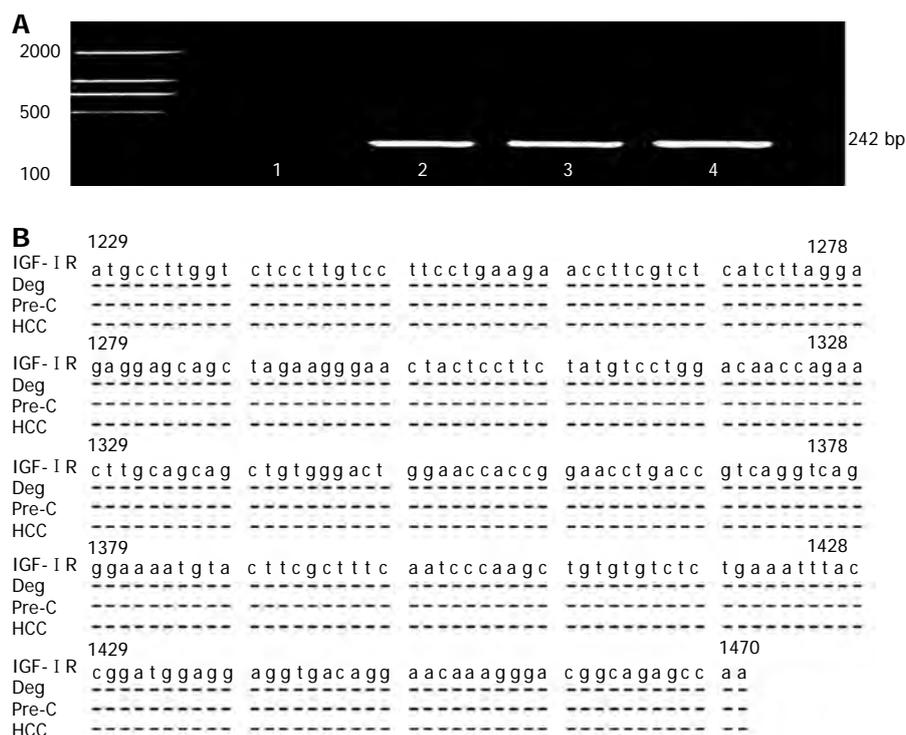
HCC: Hepatocellular carcinoma.

## RESULTS

### Pathological morphological alteration of livers

The morphological changes of rat livers in hepatocarcinogenesis induced with 2-FAA are shown in Table 1 and Figure 1. The morphological alteration of liver in rat hepatocarcinogenesis (Figure 1A-C) was confirmed by

HE staining (Table 1; Figure 1D-F), and the model rats were divided into 4 groups: the control group ( $n = 12$ ), the degeneration group ( $n = 18$  Figure 1D), the precancerous group ( $n = 9$ , Figure 1E), and the HCC group ( $n = 9$ , Figure 1F). The granule-like degeneration appeared in the cytoplasm and a large heterogeneous nucleus was seen occasional (the degeneration group, Figure 1A and D) at an early stage. At the intermediate stage, some areas had the trend to form nodules, hepatic plate cell layers increased, focal cell layers surpassed three, the nuclear chromatin was denser, and the ratio of nucleus to cytoplasm increased (the precancerous group, Figure 1B and E). The nodular hyperplasia at the later stage was observed in many areas, the structure of hepatic tissue disappeared, the hepatic cells arranged into nest or funicular form, the medium large and the nuclear chromatin was more dense, and the ratio of nucleus to cytoplasm increased, and all were confirmed as well differentiated HCC (the HCC group, Figure 1C and F), indicated that histological changes in hepatocytes from granule-like degeneration to precancerous and HCC, and confirmed by the immunohistochemistry of corresponding stage hepatic GGT



**Figure 2** Gene amplification, sequencing and homological analysis of liver insulin-like growth factor I receptor. A: The amplified fragments of rat insulin-like growth factor I receptor (IGF- I R) mRNA by reverse transcription-nested polymerase chain reaction 1, no positive fragments from control rat liver 2, the positive fragments from degeneration rat (Deg) liver; 3, the positive fragments from precancerous rat (Pre-C) liver, and 4, the positive fragments from hepatocellular carcinoma (HCC) rat liver; B: The amplified fragment of rat IGF- I R mRNA was confirmed by sequencing and alignment of nucleotide sequences from rat liver.

**Table 2** Total RNA and insulin-like growth factor- I receptor mRNA expression in hepatocarcinogenesis *n* (%)

Group	<i>n</i>	Total RNA level			IGF- I R mRNA	
		( $\mu\text{g}/\text{mg}$ wet liver)	<i>q</i>	<i>P</i> value	Positive	<i>P</i> value
Control	12	1.58 $\pm$ 0.94			0 (0.0)	
Degeneration	18	1.91 $\pm$ 0.60 <sup>1</sup>	1.177	0.249	11 (61.1) <sup>1</sup>	< 0.001
Precancerosis	9	2.00 $\pm$ 0.21 <sup>1</sup>	1.308	0.206	9 (100) <sup>1</sup>	< 0.001
HCC	9	2.86 $\pm$ 0.60 <sup>1</sup>	3.843	0.001	9 (100) <sup>1</sup>	< 0.001

<sup>1</sup>Compared with the control group. IGF- I R: Insulin-like growth factor- I receptor; HCC: Hepatocellular carcinoma.

(Figure 1G-I).

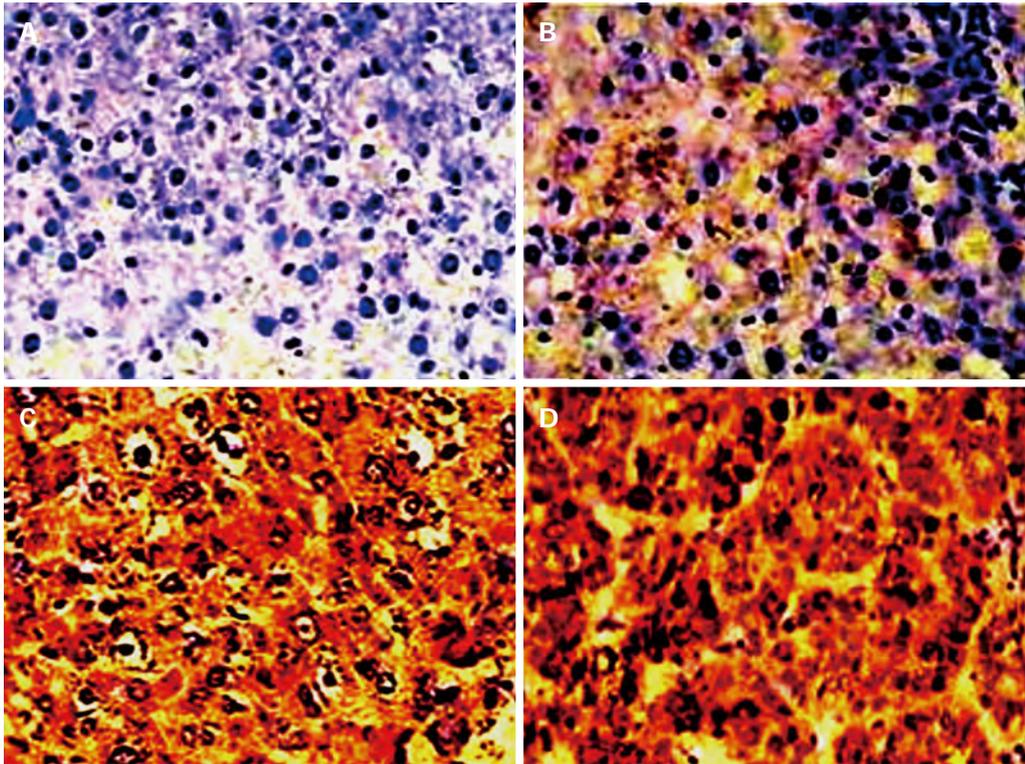
**Expression of hepatic IGF- I R mRNA**

The quantitative analysis of total liver RNA from the different stages and the positive fragments of liver IGF- I R mRNA amplification in hepatocarcinogenesis are shown in Table 2 and Figure 2. The metabolic levels of hepatic nucleic acid were vigorous and the expression of hepatic total RNA was progressively increased from the control to HCC. The specific concentration of total RNA ( $\mu\text{g}/\text{mg}$  wet liver) was significantly higher in the HCC group than that in the control group ( $F = 6.840, P < 0.001$ , Table 2). The amplified fragments of hepatic IGF- I R mRNA could be detected clearly in the HCC, precancerous, or part of degeneration group (Figure 2A) and the IGF- I R gene fragments were confirmed by sequencing (Figure 2B). The expression of IGF- I R mRNA was

progressively increased in hepatocarcinogenesis and the incidence was 0% in the control, 61.1% in the degeneration, 100% in the precancerous, and 100% in the HCC group (Table 2), respectively.

**Dynamic expression of hepatic IGF- I R**

The positive with brown particles and cellular distribution of liver IGF- I R expression analyzed by immunohistochemistry is shown in Figure 3 and the result is summarized in Table 3. The immunohistochemistry evidences indicated the positive IGF- I R expression and hepatocyte distribution of IGF- I R positive expression increased gradually along with the occurrence (Figure 3) of hepatocyte malignant transformation. The nucleus was stained in some atypical hyperplasia areas; while near the area of necrosis, the cells were mainly stained in cytoplasm and nucleus, and the positive cells were always



**Figure 3** Immunohistochemical analysis of rat liver insulin-like growth factor I receptor at the different stage of rat hepatocyte malignant transformation ( $\times 200$ ). A: No positive staining in the liver from control rat; B: The weaker insulin-like growth factor I receptor (IGF- I R) expression in the liver from degeneration rat; C: The significantly IGF- I R expression in the liver from precancerous rat; D: The over-expression of liver IGF- I R in hepatoma rats.

located in the edge of the portal area or central vein area (Figure 3C or D), and the IGF- I R levels in the precancerous or HCC group were significantly higher than that in the control group (Table 3,  $P < 0.001$ ). Of the 18 cases with degeneration, the IGF- I R-expression was detected in 9 cases and 5 of them showed moderately or strongly positive; of the 9 cases with HCC, IGF- I R over-expression was detected in 9 cases and 8 of them showed moderately or strongly positive expression (Table 3).

#### Quantitative analysis of circulating and liver IGF- I R

The levels of circulating or liver IGF- I R expression during hepatocyte malignant transformation are shown in Table 4. There was a rising tendency of serum or liver IGF- I R along with the morphological changes in hepatocarcinogenesis with HCC > precancerous > degeneration > control. The average serum IGF- I R level in the precancerous group was significantly higher than that in the control or degeneration group ( $P < 0.01$ ); and the level in the HCC group was significantly higher than that in the control or degeneration group ( $F = 11.850$ ,  $P < 0.001$ ); The IGF- I R level in the livers in the HCC group was obviously higher than that in the control, degeneration or precancerous groups ( $F = 8.720$ ,  $P < 0.001$ ). Moreover, there was a positive correlation ( $r = 0.91$ ,  $t = 14.222$ ,  $P < 0.001$ ) found between serum and liver IGF- I R, suggesting that the over-expression of liver IGF- I R release into blood and circulating IGF- I R monitor hepatocyte malignant transformation.

## DISCUSSION

HCC is one of the most common cancers and causes of mortality<sup>[20,21]</sup>. A great deal of progress in understanding the mechanism(s) of hepatocarcinogenesis has been achieved in recent years<sup>[16,22]</sup>. IGFs imbalance affect the malignant transformation of hepatocytes, and the over-expressions of IGF- II and IGF- I R promote mitosis and cellular transformation and inhibiting apoptosis<sup>[10]</sup>. IGF- I R is a trans-membrane tyrosine kinase receptor located on chromosome 15q26.3 composed of two  $\alpha$  and two  $\beta$  subunits linked by disulfide bonds. The extracellular  $\alpha$  subunit is responsible for ligand binding, whereas the  $\beta$  subunit consists of a trans-membrane domain and a cytoplasmic tyrosine kinase domain. It is always expressed efficiently in rat nonparenchymal cells including Kupffer cells, stellate cells and increases the sensitivity of the hepatocytes to IGFs mitogenic effect. In this study, the dynamic models of HCC were applied to investigate the features of IGF- I R expression in hepatocarcinogenesis, the relationship between IGF- I R expression and hepatocytes malignant transformation, and explore the multistage pathogenesis of HCC.

Hepatoma models were successfully induced in male SD rats for investigating the dynamic expression and alteration features of IGF- I R and IGF- I R-mRNA during hepatocytes malignant transformation (Table 1). At an early stage, the morphological changes of hepatocytes showed the granule-like degeneration appeared in the

**Table 3 Dynamic alteration of liver insulin-like growth factor- I receptor expression in hepatocarcinogenesis *n* (%)**

Group	<i>n</i>	Positive	<i>P</i> value	IGF- I R intensity				Nemenyi test	<i>P</i> value
				-	+	++	+++		
Control	12	0 (0.0)		12	0	0	0		
Degeneration	18	9 (50.0) <sup>1</sup>	0.004	9	4	4	1	13.716 <sup>1</sup>	< 0.050
Precancerosis	9	8 (88.9) <sup>1</sup>	< 0.001	1	3	2	3	16.229 <sup>1</sup>	< 0.001
HCC	9	9 (100) <sup>1</sup>	< 0.001	0	1	5	3	16.299 <sup>1</sup>	< 0.001

<sup>1</sup>Compared with the control group by the Kruskal-Wallis test (HC = 25.5149, *P* < 0.001). IGF- I R: Insulin-like growth factor- I receptor; HCC: Hepatocellular carcinoma.

**Table 4 Insulin-like growth factor- I level in rat livers or sera during malignant transformation of hepatocytes**

Group	<i>n</i>	Serum IGF- I R			Liver IGF- I R		
		nmol/mL	<i>q</i>	<i>P</i> value	nmol/mg wet liver	<i>q</i>	<i>P</i> value
Control	12	1.03 ± 0.47			0.43 ± 0.17		
Degeneration	18	1.51 ± 0.46 <sup>1</sup>	1.486	0.318	0.55 ± 0.20 <sup>1</sup>	0.456	0.812
Precancerosis	9	1.92 ± 0.29 <sup>1</sup>	4.116	0.003	0.66 ± 0.14 <sup>1</sup>	0.578	0.748
HCC	9	2.43 ± 0.57 <sup>1</sup>	7.674	< 0.001	0.96 ± 0.09 <sup>1</sup>	6.357	< 0.001

<sup>1</sup>Compared with the control group. IGF- I R: Insulin-like growth factor- I receptor; HCC: Hepatocellular carcinoma.

cytoplasm and a large heterogeneous nucleus occasionally. At the intermediate stage, the nuclear chromatin was denser and the ratio of nucleus to cytoplasm increased. And at the later stage, the structure of hepatic tissue disappeared, the medium large and the nuclear chromatin was denser, and the ratio of nucleus to cytoplasm increased (Figure 1). Hepatocytes were transformed into malignant cells with a large synthesis of nucleic acid. Moreover, at the stage of precancerous to hepatoma formation with the progressing increasing of liver total RNA and IGF- I R mRNA were significantly higher than those in the control or degeneration group (Table 2 and Figure 2), indicated that the activation and transcription of hepatoma related genes be closely connected with nucleic acid synthesis and IGF- I R participate in hepatocarcinogenesis<sup>[23,24]</sup>.

Significant abnormalities of IGF- I R gene transcription and expression could be detected during the process of hepatocytes malignant transformation. Recent researches showed that HCC include the over-expression of IGF- II<sup>[13]</sup>, which have close relationship with the degree of differentiation<sup>[25]</sup>. It exerts its actions mainly *via* IGF- I R. The combination then leads to activation of the phosphatidylinositol3-kinase and the Ras/mitogen activated protein kinase pathways and contributes to mitosis, proliferation, transformation and anti-apoptosis. IGF- II mRNA is over-expressed at early stage of HCC formation<sup>[13]</sup>. Immunohistochemistry of dynamic models revealed that the expression levels of IGF- I R in rat hepatic tissues and sera assumed a rising tendency during the development of HCC (Table 3, Figure 3). The average level of IGF- I R from the precancerous groups was higher than that in the normal and degeneration groups.

The over-expression of liver IGF- I R release into the blood and circulating IGF- I R could be detected (Table 4), suggesting that HCC is a high-IGF- I R expressing tumor along with the malignant transformation of hepatocytes and its abnormality should be a potential molecular marker for HCC development<sup>[26,27]</sup>.

HCC has exhibit numerous genetic abnormalities as well as epigenetic alterations including modulation of DNA methylation. Molecular factors are involved in the process of HCC development and metastasis. Several laboratories have implicated constitutive activation of miRNA as one of the early key events involving in neoplastic progression of the liver. Recent studies *in vivo* revealed that interfering with signaling *via* IGF- I R has an antitumor effect by inducing apoptosis and inhibiting cell growth<sup>[13,28]</sup>. Further studies will permit us to analyze mechanism of human hepatocarcinogenesis and pay attention to these areas. However, the combination of pathological features and some biomarkers with high sensitivity and specificity for early diagnosis of HCC seems to be more practical up to present.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death, especially in the inshore area of the Yangtze River. Insulin-like growth factor (IGF) signaling is specifically required for HCC progression, especially in IGF- II and IGF- I receptor (IGF- I R) which are the very important signaling contributors.

### Research frontiers

IGF- I R is an important regulatory factor necessary for cell growth and differentiation, provides mitogenic signal for tumor growth, exhibits a very potent anti-apoptotic activity, induces vascular endothelial growth factor expression, and able to maintain the cell phenotype transformation in carcinogenesis. However,

the features of IGF- I R expression during hepatocytes malignant transformation have not yet been reported.

### Innovations and breakthroughs

The dynamic models of rat hepatoma were applied to investigate the features of IGF- I R expression in hepatocarcinogenesis for the first time. The IGF- I R expression was progressively increased during hepatocyte malignant transformation at mRNA or protein level, and there was a positive correlation between sera and liver IGF- I R, indicating that the IGF- I R over-expression in liver tissues release into blood and circulating IGF- I R monitor occurrence of rat hepatoma.

### Applications

Overexpression of IGF- I R might participate in rat hepatocarcinogenesis and its abnormality should be an early molecular marker for hepatocyte malignant transformation.

### Peer review

This paper was aimed at investigating the expression of IGF- I R during hepatocarcinogenesis induced by 2-fluorenylacetylacetamide in Sprague-Dawley rats. Increase in IGF- I R mRNA and protein incidence and expression are reported in preneoplastic and neoplastic tissue. It is an interesting animal model study for the pathway of the IGF- I R in the HCC.

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## Endoscopic retrograde cholangiopancreatography and laparoscopic cholecystectomy during the same session: Feasibility and safety

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### Abstract

**AIM:** To explore the feasibility and safety of endoscopic retrograde cholangiopancreatography and laparoscopic cholecystectomy (LC) performed during the same session.

**METHODS:** Between July 2010 and May 2013, 156 patients with gallstones and common bile duct (CBD) stones were enrolled in this retrospective study. According to the sequence of endoscopic procedures and LC, patients were classified into two groups: in group 1, patients underwent endoscopic stone extraction and LC during the same session, and in group 2, patients underwent LC at least 3 d after endoscopic stone extraction. Outcomes of the endoscopic procedures and LC were compared between the two groups, respectively.

**RESULTS:** There were 91 patients in group 1 and 65 patients in group 2. The characteristics of the two groups were similar. The mean duration of the endoscopic procedures was 34.9 min in group 1 and 35.3

min in group 2. There were no significant differences in the success rate of the endoscopic procedures (97.8% for group 1 vs 98.5% for group 2), the total rate of endoscopic complications (4.40% for group 1 vs 4.62% for group 2) and CBD stone clearance rate (96.7% for group 1 vs 96.9% for group 2). Duration of LC was 53.6 min in group 1 and 52.8 min in group 2. There were no significant differences in the overall LC-related morbidity and postoperative hospital stay.

**CONCLUSION:** Endoscopic stone extraction and LC performed during the same session was feasible and safe in patients with gallstones and concomitant CBD stones.

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**Key words:** Cholecystectomy; Laparoscopic; Endoscopic; Therapy

**Core tip:** There is still controversy regarding the optimal therapeutic algorithm for patients with gallstones and concomitant common bile duct stones. Endoscopic retrograde cholangiopancreatography combined with laparoscopic cholecystectomy is an alternative technique. This study originated from a surgical team adept in the techniques of laparoscopy, duodenoscopy and choledochoscopy. The sample size in this study was larger than most current studies. We are confident that the outcomes from this study are more objective and helpful to the surgeons who manage gallstones and common bile duct stones during the same session.

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## INTRODUCTION

Approximately 5% to 15% of patients with gallstones have concomitant common bile duct (CBD) stones<sup>[1]</sup>. As the debate on the optimal choice for these patients is ongoing, surgeons are often bewildered by the variety of therapies which have emerged in the minimally invasive era. Laparoscopic cholecystectomy (LC) combined with endoscopic clearance of CBD stones is an appropriate option. Early LC after endoscopic CBD stone extraction is currently considered a good algorithm<sup>[2-5]</sup>. Reports of endoscopic stone extraction and LC being performed during the same session have continued to rise since LC became the standard procedure for cholecystectomy<sup>[6,7]</sup>. Many efforts have been made to develop this type of combination and the results are encouraging<sup>[8-15]</sup>.

If endoscopic stone extraction and LC are to be performed during the same session as routine practice, the feasibility and safety should be investigated objectively. In the present study, we compared this method with the current practice where LC is performed after endoscopic stone extraction. Our surgical team is adept in the techniques of laparoscopy duodenoscopy and choledochoscopy, and the indications for the use of these minimally invasive procedures were stricter and more objective. In addition, this study provided more details on the combination of laparoscopic and duodenoscopic therapy during the same session.

## MATERIALS AND METHODS

In this retrospective study, 156 patients with gallstones and CBD stones underwent endoscopic stone extraction and LC performed by the same surgical team at Taizhou People's Hospital (Taizhou, Jiangsu Province, China) between July 2010 and May 2013. The study was approved by our ethics committee, and written consent was obtained from each patient. After being informed about the related therapeutic maneuver, the patients chose the sequence of endoscopic procedure and LC. According to this sequence, the patients were classified into two groups: in group 1, patients underwent endoscopic stone extraction and LC during the same session, and in group 2, patients underwent LC at least 3 d after endoscopic stone extraction<sup>[5]</sup>. Dr. Zang JF, who has significant experience in manipulation of the duodenoscope, laparoscope and choledochoscope, performed all main endoscopic and laparoscopic procedures in this study.

In group 1, general anesthesia with intubation from mouth was chosen in all patients. The endoscopic retrograde cholangiopancreatography (ERCP) procedure was performed with the patients in the prone position. A duodenoscope (TJF160R, Olympus, Japan) was inserted into the duodenal second segment *via* the mouth. A cholangiogram was carried out using C-arm X-ray (BV-Libra, Philips) and an endoscopic sphincterotomy (EST) was performed to extract the CBD stones. The stones were removed by basket or balloon catheter. Stones larger than 10 mm were removed using a mechanical lithotripter. A

**Table 1 Characteristics of the patients**

Characteristic	Group 1 (n = 91)	Group 2 (n = 65)
Age (yr)	53.6 ± 8.7	55.3 ± 9.4
Sex (male/female)	32/59	17/48
ASA (I / II / III)	63/23/5	12/4/19/49
Diameter of CBD (mm)	10.5 ± 2.6	11.2 ± 2.7
Maximal diameter of CBD stone (mm)	6.4 ± 1.4	6.7 ± 1.3
Bilirubin (μmol/L)	37.3 ± 8.6	39.1 ± 9.2
Alanine aminotransferase (U/L)	127.2 ± 43.5	119.5 ± 47.8
Aspartate aminotransferase (U/L)	105.9 ± 32.7	109.8 ± 35.0
Gamma glutamyl transpeptidase (U/L)	224.3 ± 63.3	233.1 ± 65.1

CBD: Common bile duct; ASA: American Society of Anesthesiologists.

nasobiliary drain was inserted if there was a possibility of retained stones. This allowed sequential cholangiography and irrigation. Following ERCP, care was taken to remove all the gas from the stomach to facilitate LC. The patients were then placed in the reverse Trendelenburg position. LC was performed using the three trocar technique. A subhepatic drain was positioned if there was concern about the anatomy of the cystic duct and a danger of bleeding.

In group 2, patients underwent endoscopic stone extraction with general anesthesia by intubation. If there were no complications related to ERCP, LC was performed three d later. Otherwise, LC was delayed until the ERCP-related complications were successfully treated.

### Statistical analysis

Medical records, endoscopic and operative reports were reviewed. The statistical data were analyzed using *t* test, Pearson's  $\chi^2$  or Fisher's exact test. The results were analyzed using a statistical analysis program (SPSS 16.0). *P* < 0.05 was considered statistically significant.

## RESULTS

### Patients' characteristics

The characteristics of the two treatment groups, including main pre-operative biochemical data, are shown in Table 1. No significant differences were identified with respect to age, gender and other medical conditions.

### Outcomes of endoscopic procedures

During the same period, two patients undergoing ERCP in group 1 and one patient in group 2 converted to laparoscopic common bile duct exploration (LCBDE) due to unsuccessful bile duct cannulation. There was no significant difference in the success rate of endoscopic procedures (97.8% for group 1 *vs* 98.5% for group 2; Fisher's exact test *P* = 1.000). The mean duration of endoscopic procedures was 34.9 min in group 1 and 35.3 min in group 2. Pancreatitis was observed in 2 patients in each group. EST-related hemorrhage occurred in one patient in group 1 and one patient in group 2. One patient in group 1 experienced cholangitis. The total rates of endoscopic complications were 4.40% in group 1 and 4.62% in group

**Table 2** Outcomes of endoscopic procedures, laparoscopic cholecystectomy

	Group 1 (n = 91)	Group 2 (n = 65)
Endoscopic procedures		
Success rate	97.80%	98.50%
Time (min)	34.9 ± 10.9	35.3 ± 9.7
Complications	4.40%	4.62%
Pancreatitis (n)	2	2
Hemorrhage (n)	1	1
Cholangitis (n)	1	0
Nasobiliary drainage (n)	11	8
CBD stone clearance	96.70%	96.90%
Laparoscopic cholecystectomy		
Operation duration (min)	53.6 ± 12.1	52.8 ± 13.4
Subhepatic drainage (n)	6	5
Time to flatus (h)	17.5 ± 6.7	18.3 ± 7.1
Complications (n)		
Infection	1	1
Bleeding	1	0
Conversion	1	1
Hospital stay (d)		
Postoperative	3.13 ± 0.96	2.95 ± 1.08
Total (P < 0.01)	5.32 ± 1.26	9.27 ± 1.31

CBD: Common bile duct.

2 (Fisher's exact test,  $P = 1.000$ ). CBD stone clearance rates (96.7% for group 1 *vs* 96.9% for group 2; Fisher's exact test  $P = 1.000$ ) were not significantly different. Complications of ERCP were treated conservatively. Outcomes of endoscopic procedures are shown in Table 2.

### Outcomes of laparoscopic cholecystectomy

Mortality was not observed in the treatment groups. Duration of surgery was 53.6 min in group 1 and 52.8 min in group 2 ( $P = 0.70$ ). The overall LC-related morbidity (including conversion) was 3/91 in group 1 and 2/65 in group 2 (Fisher's exact test,  $P = 1.000$ ). Bile duct injury was not observed in this study. The median time to flatus was 17.5 h in group 1 and 18.3 h in group 2 ( $P = 0.47$ ). There was no significant difference in postoperative hospital stay between the groups ( $P = 0.27$ ). The total length of hospital stay in group 1 was shorter than that in group 2 (5.32 ± 1.26 d in group 1 *vs* 9.27 ± 1.31 d in group 2,  $P < 0.001$ ). The outcomes of LC are shown in Table 2.

## DISCUSSION

As therapies for patients with gallstones and CBD stones are discussed in the current era of minimal invasion, biliary surgeons are required to master more techniques than ever before. Following the development of endoscopic techniques over the last three decades, these techniques have been confirmed as efficient and safe in the treatment of CBD stones. However, the diagnostic value of these methods in biliary diseases, especially benign diseases, has decreased markedly due to inherent invasion. Magnetic resonance cholangio pancreatography has gradually become an alternative and is considered to be a noninvasive diagnostic technique in biliary diseases<sup>[16-18]</sup>. ERCP combined with LC is widely used to treat gallstones with con-

comitant CBD stones. However, ERCP and LC are often performed by two separate surgical teams, which results in difficulties when these two procedures are carried out during the same session. In this study, ERCP and LC were performed by the same surgical team as a routine procedure. Therefore, the outcomes are more convincing and objective than many other reports<sup>[9,10]</sup>.

The feasibility of endoscopic CBD stone extraction and LC during the same session is a problem for surgeons. Although this protocol was proposed twenty years ago, only a few medical faculties have carried out this procedure. A likely reason for this is that there is no specific standard process to refer to. In addition, the technique is difficult, especially when choosing the patient's position in ERCP and managing distension of the intestine due to air insufflation. Initially, patients were placed in the supine position. However, three problems forced us to change this: difficulty in locating the endoscopic tip in the second portion of the duodenum, trouble with positioning the papilla correctly and repeated interference due to liquid in the duodenal cavity. In our view and that of ElGeidie<sup>[13]</sup>, the disadvantages of the supine position advocated by Terruzzi *et al*<sup>[19]</sup> may not be easily resolved due to the inherent anatomy of the papilla, which is located in the posterior medial duodenal wall. In comparison with the supine position, the prone position is our favored position which is optimal for cannulation of the papilla and obtaining good-quality radiographic images. Furthermore, the prone position provides a natural pressure due to the weight of the abdomen, restricting distension of the intestine. General anesthesia with tracheal intubation can reduce patients' discomfort and make airway management easier. The only problem with the prone position was that it took operators a few minutes to change the patient's position twice. In addition, we insufflated the least amount of air during ERCP and suctioned the duodenal and gastric cavity thoroughly at the end of ERCP. It is also important that endoscopists control the time of ERCP by prudent preoperative evaluation and exact intraoperative judgment, including optimal timing of conversion therapy. We selected patients in whom the maximal diameter of stones was less than 15 mm. Thus, the average duration of our endoscopic procedure was approximately 35 min. Using this protocol, ERCP was not an obstacle to subsequent LC if more attention was paid to the choice of patients and manipulation of ERCP.

The safety of the combination of laparoscopic and endoscopic techniques during the same session is also a focus of concern for surgeons. Currently, morbidity and mortality in endoscopic and laparoscopic procedures are very low with skilled operators. There are no disputes when LC and endoscopic CBD stone extraction are discussed. In the present study, there were no differences in complication rates and patients' recovery between the two treatment groups, which demonstrated that this combination was safe. In order to reduce ERCP-related complications, many authors have used the laparo-endo-

scopic rendezvous technique which was considered helpful in cannulation of the bile duct and preventing post-ERCP pancreatitis<sup>[10,11,15]</sup>. However, this technique failed as the endoscopist performed ERCP with the patient in the supine position and the guidewire was unable to pass through the cystic duct. As proficient endoscopists, we regard the rendezvous technique as a transitional method and not the ultimate method. With the help of a guidewire and needle-knife, the overall success of bile duct cannulation was 97% and the mean time to bile duct access was 10 min according to our records. The rate of post-ERCP pancreatitis, which was mild in most patients, was 4% in our hospital. Direct ERCP during surgery is more likely to become the mainstream maneuver rather than the rendezvous technique. LC with endoscopic stone extraction during the same session could be considered a safe procedure as the rate of LC-related complications, including bile duct injury and conversion, was low in our hospital. This was consistent with our previous results which showed that the earlier LC is performed after the endoscopic procedure, the better the outcome will be<sup>[5]</sup>.

With regard to same-session surgery for patients with concomitant gallstones and choledocholithiasis, laparoscopic common bile duct exploration (LCBDE) is another good choice rather than the combination of duodenoscopy and laparoscopy. These techniques have similar primary ductal clearance rates and morbidity<sup>[20,21]</sup>. Some studies have shown that LCBDE is more cost-effective<sup>[21,22]</sup> and more beneficial in the preservation of papillary function than ERCP, although suffering due to T-tube placement is a major disadvantage. Difficulties in LCBDE include extraction of the CBD stone and suturing of the CBD incision. With regard to the indications for LCBDE and endoscopic stone extraction, a clear consensus has emerged although different surgeons have different opinions. A CBD with a diameter smaller than 8 mm is regarded as a contraindication to LCBDE. Similarly, large stones may be a barrier to duodenoscopy. Although endoscopic extraction of large stones (even 25 mm) has been reported, we prefer to perform LCBDE in these cases (stone diameter  $\geq$  15 mm). Laser lithotripsy by choledochoscope in LCBDE may be more direct and easier for the management of large stones. For patients who were fit for both procedures, the final choice involved many aspects: patients' desire, hospital resources, and surgeons' technique. The long-term effects of endoscopic sphincterotomy include recurrent stones, cholangitis, and cholangiocarcinoma, however, current evidence may partly dispel these associations<sup>[23,24]</sup>.

In conclusion, endoscopic stone extraction and LC performed during the same session is feasible and safe in patients with gallstones and concomitant CBD stones. We propose that surgeons mastering endoscopic and laparoscopic techniques may attempt this procedure. More objective studies including a comparison of this modality and LCBDE, especially the long-term effects, are urgently needed.

## COMMENTS

### Background

Approximately 5% to 15% of patients with gallstones have concomitant common bile duct stones. The debate on the optimal treatment choice for these patients is ongoing, and surgeons are often bewildered by the variety of therapies emerging in the minimally invasive era. Endoscopic retrograde cholangiopancreatography (ERCP) and laparoscopic cholecystectomy (LC) performed during the same session have been reported in many studies with encouraging results. However, most available data have come from surgeons who have usually mastered one technique either ERCP or LC. Available studies on this combined therapeutic modality included small sample sizes.

### Research frontiers

Reports on endoscopic stone extraction and LC performed during the same session have continued to increase since LC became the standard procedure for cholecystectomy. If endoscopic stone extraction and LC are to be performed during the same session as routine practice, the feasibility and safety should be investigated. Although this protocol was proposed twenty years ago, no specific standard practice has been established. Morbidity and mortality in both endoscopic and laparoscopic procedures are very low with skilled operators. The safety of combining laparoscopic and endoscopic techniques during the same session should be evaluated before it becomes a routine procedure.

### Innovations and breakthroughs

This method was compared with the method used in current practice, laparoscopic cholecystectomy after endoscopic stone extraction. Their surgical team is adept in the techniques of laparoscopy, duodenoscopy and choledochoscopy, and the indications for use of these minimally invasive procedures are stricter and more objective. In addition, this study provided more details on the combination of laparoscopic and duodenoscopic therapy during the same session.

### Applications

The outcomes of this study are more objective for evaluating the effect of ERCP/LC on gallstones and concomitant common bile duct (CBD) stones. Also, it offers surgeons more choice when they want to manage gallstones and CBD stones during the same session.

### Peer review

ERCP and LC performed during the same session is a good choice for patients with gallstones and concomitant CBD stones. This study originated from a surgical team, which is adept in the techniques of laparoscopy, duodenoscopy and choledochoscopy. It is helpful for surgeons to manage gallstones and common bile duct stones during the same session.

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## *Helicobacter pylori* infection and esophageal cancer risk: An updated meta-analysis

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### Abstract

**AIM:** To clarify the association between *Helicobacter pylori* (*H. pylori*) infection and the risk of esophageal carcinoma through a meta-analysis of published data.

**METHODS:** Studies which reported the association between *H. pylori* infection and esophageal cancer published up to June 2013 were included. The odds ratios (ORs) and corresponding 95%CIs of *H. pylori*

infection on esophageal cancer with respect to health control groups were evaluated. Data were extracted independently by two investigators and discrepancies were resolved by discussion with a third investigator. The statistical software, STATA (version 12.0), was applied to investigate heterogeneity among individual studies and to summarize the studies. A meta-analysis was performed using a fixed-effect or random-effect method, depending on the absence or presence of significant heterogeneity.

**RESULTS:** No significant association between *H. pylori* infection and esophageal squamous cell carcinoma (ESCC) risk was found in the pooled overall population (OR = 0.97, 95%CI: 0.76-1.24). However, significant associations between *H. pylori* infection and ESCC risk were found in Eastern subjects (OR = 0.66, 95%CI: 0.43-0.89). Similarly, cytotoxin-associated gene-A (CagA) positive strains of infection may decrease the risk of ESCC in Eastern subjects (OR = 0.77, 95%CI: 0.65-0.92), however, these associations were not statistically significant in Western subjects (OR = 1.26, 95%CI: 0.97-1.63). For esophageal adenocarcinoma (EAC) the summary OR for *H. pylori* infection and CagA positive strains of infection were 0.59 (95%CI: 0.51-0.68) and 0.56 (95%CI: 0.45-0.70), respectively.

**CONCLUSION:** *H. pylori* infection is associated with a decreased risk of ESCC in Eastern populations and a decreased risk of EAC in the overall population.

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**Key words:** *Helicobacter pylori*; Esophageal carcinoma; Cancer risk; Meta-analysis

**Core tip:** Based on this meta-analysis, we found that *Helicobacter pylori* infection may have a protective effect against esophageal squamous cell carcinoma in

Eastern populations and against esophageal adenocarcinoma in the overall population.

Xie FJ, Zhang YP, Zheng QQ, Jin HC, Wang FL, Chen M, Shao L, Zou DH, Yu XM, Mao WM. *Helicobacter pylori* infection and esophageal cancer risk: An updated meta-analysis. *World J Gastroenterol* 2013; 19(36): 6098-6107 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6098.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6098>

## INTRODUCTION

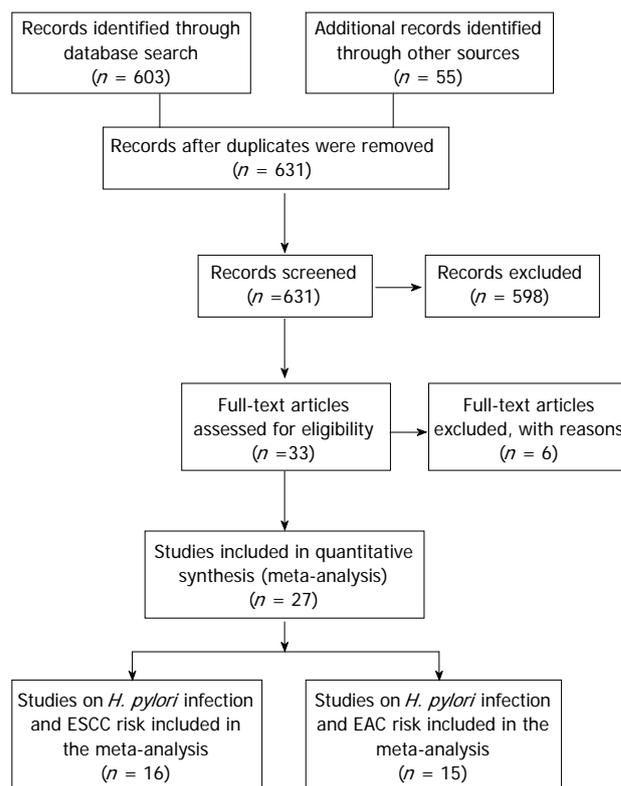
Esophageal cancer (EC), which mainly consists of esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), is the eighth most common cancer worldwide, with 481645 new cases in 2008, and is the sixth leading cause of cancer death, with 406533 deaths<sup>[1]</sup>. Despite advances in the molecular mechanism of carcinogenesis, the etiology of this malignancy remains unclear. A better understanding of the influencing factors and underlying mechanisms involved in EC development and progression will provide appropriate targets for the development of effective strategies for the prevention of this prevalent malignancy.

*Helicobacter pylori* (*H. pylori*) is a helical-shaped Gram-negative bacterium and has been identified as the major causative agent of various benign and malignant gastrointestinal tract diseases<sup>[2]</sup>. A study showed that cytotoxin-associated gene-A (CagA)-positive strains conferred a greater risk than CagA-negative strains<sup>[3]</sup>. Islami *et al*<sup>[4]</sup> carried out an excellent meta-analysis and reported an inverse association between CagA-positive *H. pylori* colonization and the risk of EAC, but not ESCC. However, recent studies reported inconclusive results, and some showed the reverse relationship between *H. pylori* and ESCC<sup>[5,6]</sup>. Therefore, an updated meta-analysis was performed which included all eligible studies to evaluate the association between *H. pylori* infection and EC risk.

## MATERIALS AND METHODS

### Search strategy

To identify all articles that examined the association between *H. pylori* infection and esophageal carcinoma, we conducted a literature search in the PubMed databases up to April 2013 using the following MeSH terms and keywords: “*Helicobacter pylori*” [MeSH] OR (*Campylobacter pylori*) OR (*H. pylori*) OR (*H. pylori*) AND (“Esophageal Neoplasms” [MeSH] OR (Cancer of Esophagus) OR (Cancer of the Esophagus) OR (Esophageal Cancer) OR (Esophagus Cancer) OR (Esophagus Neoplasm) OR (Neoplasms, Esophageal)). Additional studies were identified by a hand search from references of original studies or review articles on this topic. Two authors reviewed the search results to reduce the possibility of missing relevant published papers. Where data were missing, we contacted the authors for the



**Figure 1** Flow diagram of study identification. ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma; *H. pylori*: *Helicobacter pylori*.

relevant information. Eligible studies included in this meta-analysis had to meet the following criteria: (1) Articles should clearly describe studies on the association between *H. pylori* infection and esophageal cancer risk; and (2) The esophageal cancer diagnoses and the sources of cases and controls should be stated. The literature excluded in this study was mainly due to the following reasons: lacking a normal control group, reviews, the research design being not scientific and reasonable, and including repeated data. A total of 27 publications met the eligibility criteria and were included in the present study<sup>[5-31]</sup>.

### Data extraction

The following data from each article were extracted: authors, year of publication, country of participants, study design, source of controls, number of controls and of cases, *H. pylori* detection method, and *H. pylori* infection status. The data were extracted and registered in two databases independently by two investigators (Zheng QQ and Wang FL) who were blind to journal names, institutions and funding grants. Any discrepancy between these two investigators was resolved by a third investigator (Xie FJ), who participated in the discussion and made the ultimate decision. Equivocal or missed data were excluded in order to unify the formation of the information<sup>[11,23]</sup>. One article was used as a partial adjusted value, due to crude data<sup>[10]</sup>, and 2 articles that were included totally or partially in another article<sup>[32,33]</sup>.

Table 1 Characteristics of literatures included in the meta-analysis

Ref.	Study area	Year	Study type	HP Dm	Hp <sup>+</sup> definition <sup>1</sup>	ESCC				EAC				Matched/adjusted <sup>3</sup>	Control
						Case		Control		Case		Control			
						Hp <sup>+</sup>	CagA <sup>+</sup>	Hp <sup>+</sup>	CagA <sup>+</sup>	Hp <sup>+</sup>	CagA <sup>+</sup>	Hp <sup>+</sup>	CagA <sup>+</sup>		
Murphy <i>et al.</i> <sup>[7]</sup>	Finnish	2012	Pop	S	HpSe <sup>+</sup>	64/82	35/82	63/82	36/82	-	-	-	-	Yes/yes	Matched with age, smoking and Alcohol consumption, and date of blood draw to controls, all of cases and controls were male smokers
Khoshbaten <i>et al.</i> <sup>[8]</sup>	Iran	2011	Pop	S	HpSe <sup>+</sup>	58/100	28/100	83/100	36/100	-	-	-	-	Yes/no	Sex-matched and age-matched health individuals, any clinical evidence of gastrointestinal symptoms were excluded
Venerito <i>et al.</i> <sup>[8]</sup>	Germany	2011	Clin	S, H, U	His <sup>+</sup> , HpSe <sup>+</sup> , CagA <sup>+</sup> or U <sup>+</sup>	53/75	42/75	53/75	40/75	-	-	-	-	Yes/no	Sex and age-matched individuals with upper GI symptoms but no malignancy of the upper GI tract
Whiteman <i>et al.</i> <sup>[11]</sup>	Australia	2010	Pop	S	HpSe <sup>+</sup>	54/208	-	302/1316	-	35/260	-	302/1316	-	Yes/yes	Randomly selected from the same areas, matched to each stratum of age and state
Cook <i>et al.</i> <sup>[10]</sup>	Finnish	2010	Pop	S	HpSe <sup>+</sup>	64/78	35/78	71/91	42/91	-	-	-	-	Yes/yes	Matched to case for age, years of smoking, cigarettes per day, or body mass index
Wu <i>et al.</i> <sup>[5]</sup>	Taiwan	2009	Pop	S	HpSe <sup>+</sup>	112/317	91/317	563/1103	268/700	-	-	-	-	No/yes	One part of control is matched by gender and age, but another part wasn't matched
Hu <i>et al.</i> <sup>[6]</sup>	Taiwan	2009	Pop	S	HpSe <sup>+</sup>	66/180	-	102/194	-	-	-	-	-	Yes/yes	Healthy and cancer-free individuals, matched to age, sex and ethnicity
Früh <i>et al.</i> <sup>[12]</sup>	Canada	2008	Clin	S	CagA <sup>+</sup> or VacA <sup>+</sup>	-	-	-	-	36/100	29/100	43/101	30/101	Yes/yes	Healthy GERD-free, non-blood-related family member and friends of other cancer/surgical patients
Derakhshan <i>et al.</i> <sup>[24]</sup>	Iran	2008	Clin	S	HpSe <sup>+</sup>	-	-	-	-	9/19	-	28/38	-	Yes/yes	Dyspeptic patients with no peptic ulcer or tumor in their endoscopy
Anderson <i>et al.</i> <sup>[25]</sup>	Ireland	2008	Pop	S	HpSe <sup>+</sup>	-	-	-	-	55/123	57/123	157/253	150/253	Yes/yes	Randomly selected population-based controls, frequency matched to EAC cases for age and sex
Löfdahl <i>et al.</i> <sup>[23]</sup>	Sweden	2008	Pop	S	HpSe <sup>+</sup> or CagA <sup>+</sup>	-	-	-	-	130/230	-	304/499	-	Yes/no	Random selected from the population register, frequency matched for age and sex
Anandasabapathy <i>et al.</i> <sup>[26]</sup>	United States	2007	Clin	H	His <sup>+</sup>	-	-	-	-	4/25	-	10/30	-	No/no	Barrett's patients with no dysplasia
Iijima <i>et al.</i> <sup>[27]</sup>	Japan	2007	Clin	S, H, U	HpSe <sup>+</sup> , His <sup>+</sup> or U <sup>+</sup>	60/73	-	56/73	-	-	-	-	-	Yes/yes	Endoscoped patients with no localized lesion, matched to cases for age and sex
Kamangar <i>et al.</i> <sup>[14]</sup>	China	2007	Pop	S	HpSe <sup>+</sup>	231/335	178/335	662/992	552/992	-	-	-	-	No/yes	Randomly selected from the entire baseline participants in the study cohort
Simán <i>et al.</i> <sup>[13]</sup>	Sweden	2007	Pop	S	HpSe <sup>+</sup>	15/37	24/37	68/129	82/129	4/12	6/12	24/47	32/47	Yes/yes	Randomly selected from the study cohort, matched with age, sex, and date enrollment
Wang <i>et al.</i> <sup>[28]</sup>	China	2006	Pop	S	HpSe <sup>+</sup>	?/107	-	?/107	-	-	-	-	-	Yes/yes	Neighborhood controls, randomly selected, and matched to cases for age and gender
Wu <i>et al.</i> <sup>[15]</sup>	Taiwan	2005	Pop	S	HpSe <sup>+</sup>	28/127	-	74/171	-	-	-	-	-	No/yes	Randomly selected from the same community
de Martel <i>et al.</i> <sup>[29]</sup>	United States	2005	Pop	S	HpSe <sup>+</sup>	-	-	-	-	19/51	9/18	74/150	44/71	Yes/yes	Randomly selected from the study cohort, matched with age, sex, and date enrollment rate, and study site
Ye <i>et al.</i> <sup>[16]</sup>	Sweden	2004	Pop	S	HpSe <sup>+</sup>	32/85	63/85	198/499	293/499	18/97	42/97	198/499	293/499	Yes/yes	Randomly selected population-based controls, frequency matched to EAC cases for age and sex

Author	Year	Country	Study Type	Exposure	Outcome	Healthy Subjects	Yes/no	Matched Cases	Yes/yes	Yes/no	Gender
Wang <i>et al.</i> <sup>[30]</sup>	2003	China	Pop	S	HpSe <sup>+</sup>	145/310	-	-	-	-	Healthy subjects with no difference in age and gender
Wu <i>et al.</i> <sup>[21]</sup>	2003	United States	Pop	S	HpSe <sup>+</sup>	-	49/80	230/356	106/356	-	Matched to cases for age, sex, neighborhood of residence, and race
El Omar <i>et al.</i> <sup>[21]</sup>	2003	United States	Pop	S	HpSe <sup>+</sup>	7/26	35/108	84/210	46/224	-	Matched to cases for age, sex, and study center
Weston <i>et al.</i> <sup>[20]</sup>	2000	United States	Clin	H	His <sup>+</sup>	-	3/20	96/217	-	-	Patients with GERD symptoms but no Barrett's esophagus
Vieeth <i>et al.</i> <sup>[9]</sup>	2000	Germany	Clin	H	His <sup>+</sup>	-	66/138	468/712	-	-	Patients with non-ulcer dyspepsia and no endoscopic signs of GERD
Peek <i>et al.</i> <sup>[18]</sup>	1999	United States	Clin	S, H	HpSe <sup>+</sup> or His <sup>+</sup>	-	11/30	20/48	25/48	-	Patients endoscoped for reasons other Than GERD or Barrett's
Oberg <i>et al.</i> <sup>[61]</sup>	1999	United States	Clin	H	His <sup>+</sup>	-	5/37	32/229	-	-	Patients with foregut symptoms and benign diseases
Talley <i>et al.</i> <sup>[17]</sup>	1991	United States	Clin	S	HpSe <sup>+</sup>	96/252	-	-	-	-	Asymptomatic volunteers and patients with benign esophageal, lung, orculoskeletal disorders

<sup>1</sup>*Helicobacter pylori* (*H. pylori*) positivity definition; <sup>2</sup>Because the article showed that there was no difference in age and gender between cases and health controls, this study was analyzed as a matched population study; <sup>3</sup>Individual-matching of controls to cases for age and gender/reporting adjusted ORs for the association between *H. pylori* and cancer. Clin: Clinical based; Pop: Population based; NR: Not reported; Hp Dm: Hp detection method; S: Serology; H: Histology; U: Rapid urease test; His<sup>+</sup>: Positive histological examination of tissue samples; HpSe<sup>+</sup>: Sero-positivity for antibodies to whole-cell; VacA<sup>+</sup>: Sero-positivity for antibodies to VacA; CagA<sup>+</sup>: Sero-positivity for antibodies to cytotoxin-associated gene-A; U<sup>+</sup>: Positive rapid urease test; GERD: Gastroesophageal reflux disease; ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma; GI: Gastrointestinal.

### Statistical analysis

The dichotomous data on *H. pylori* positive results in the EC group and control group were summarized. OR and 95%CI of OR were calculated to assess the association between *H. pylori* infection and EC risk. If the *H. pylori* data were not shown in the article, the OR and 95%CI: value were extracted. An analysis of the heterogeneity of the studies was performed using the  $\chi^2$ -based *Q* test. A *P* value less than 0.05 was considered significant for heterogeneity. If the studies were shown to be homogeneous with *P* > 0.05 for the *Q*-statistics, the summary of OR was calculated by a fixed-effects model (the Mantel-Haenszel method), otherwise, the random-effects model (the DerSimonian and Laird method) was used. The potential publication bias was assessed graphically using Begg's test and funnel plots. All analyses were performed with STATA software (version 12.0; Stata Corp LP, College Station, TX, United States), using two-sided *P* values.

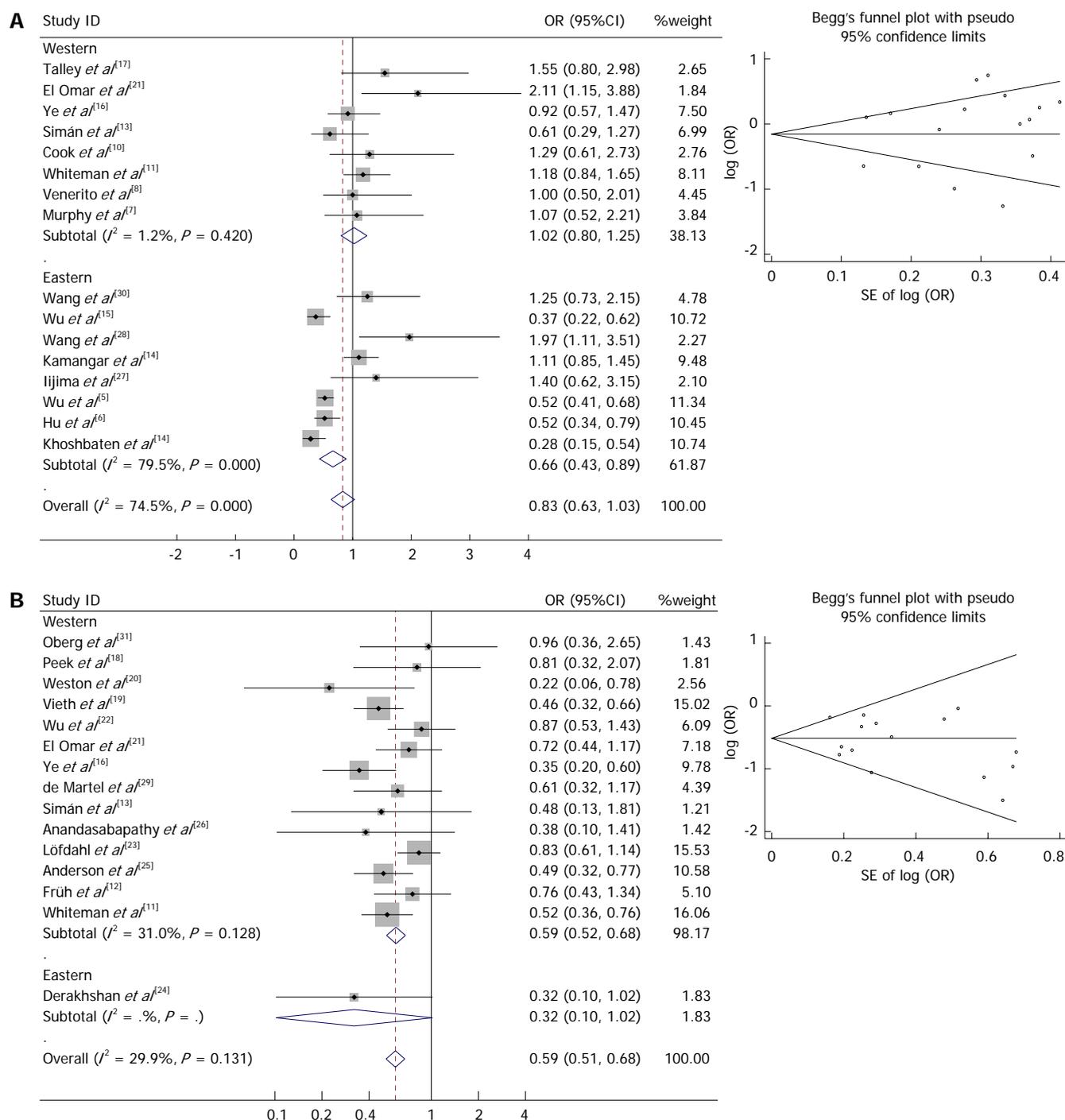
## RESULTS

### Eligible studies

Twenty-seven eligible studies on *H. pylori* infection and esophageal cancer were identified through the literature search and selection based on the inclusion and exclusion criteria (Figure 1). The year of publication for these studies ranged from 1991 to 2012. There were 18 studies on Western (Finland, Germany and Ireland) populations and 9 studies on Eastern (Iran and China) populations. With respect to study type, 17 studies were population-based, 10 studies were hospital-based and one study did not specify. Adjusted ORs with corresponding 95%CIs were reported in 17 studies. The selected study characteristics are summarized in Table 1.

### Test of heterogeneity

We analyzed the heterogeneity of all 16 studies on ESCC and the fifteen studies on EAC, respectively. For *H. pylori* infection in the ESCC risk study, the *Q* statistic was significant (*P* < 0.01) and the *I*<sup>2</sup> statistic showed a high variation (*I*<sup>2</sup> = 74.5%) among the study results, thus a random-effect model was used for further analysis (Figure 2A and Table 2). In the EAC study, no significant heterogeneity was observed in the overall comparison (*I*<sup>2</sup> = 29.9%, *P*<sub>heterogeneity</sub> = 0.131), and a fixed-effect model was used to calculate the overall ORs (Figure 2B and Table 2).



**Figure 2** Forest plot and Begg's funnel plot of the association between *Helicobacter pylori* infection and esophageal carcinoma. Studies are sorted in order of publication year. A: Esophageal squamous cell carcinoma (random-effect model); B: Esophageal adenocarcinoma (fixed-effect model).

***H. pylori* infection and ESCC risk**

The association between *H. pylori* infection and ESCC risk is shown in Figure 2A and Table 2. With the exception of the clinical-based Western studies, CagA<sup>+</sup> strains in the Eastern and Western studies did not show obvious heterogeneity calculated using the fixed-effect model. The remaining results were significantly heterogeneous ( $P < 0.01$ ) calculated using the random-effect model.

In the random-effects model, no statistically significant factor influenced the risk of ESCC in the presence

of *H. pylori* infection (OR = 0.83, 95%CI: 0.63-1.03). When population-based studies were analyzed alone, the combined OR for the association between *H. pylori* infection and ESCC risk was 2.86 (95%CI: 1.60-5.11). When clinical-based studies were analyzed alone, the combined OR for *H. pylori* infection was 1.49 (95%CI: 0.66-2.31). When stratified by study location, there was a statistically significant decrease in ESCC risk in the Eastern population (OR = 0.66, 95%CI: 0.43-0.89), however, we did not find a significant association in the Western popula-

**Table 2** Meta-analysis of the *Helicobacter pylori* infection on the risk of esophageal squamous cell carcinoma and esophageal adenocarcinoma

	Studies	<i>P</i> <sup>1</sup>	<i>I</i> <sup>2,2</sup>	Overall OR (95%CI)
Esophageal squamous cell carcinoma				
Case/control (1961/5704)				
All studies	16	< 0.01	74.50%	0.83 (0.63, 1.03)
Population-based studies	14	< 0.01	76.00%	0.79 (0.59, 1.00)
Clinical-based studies	2	0.86	< 0.01	1.49 (0.66, 2.31)
Eastern studies	8	< 0.01	79.50%	0.66 (0.43, 0.89)
Western studies	8	0.42	1.20%	1.02 (0.80, 1.25) <sup>3</sup>
Studies with matched controls	11	< 0.01	71.80%	0.90 (0.61, 1.20)
Studies without matched controls	5	< 0.01	82.90%	0.79 (0.46, 1.12)
<i>Hp</i> <sup>+</sup> only definition as <i>HpSe</i> <sup>+</sup>	14	< 0.01	76.80%	0.81 (0.60, 1.02)
Adjusted results	11	< 0.01	80.50%	0.84 (0.56, 1.12)
<i>CagA</i> <sup>+</sup> vs <i>Hp</i> <sup>-</sup>	9	0.03	52.00%	0.97 (0.76, 1.24)
Eastern study	3	0.22	35.00%	0.77 (0.65, 0.92) <sup>3</sup>
Western studies	6	0.39	3.60%	1.26 (0.97, 1.63) <sup>3</sup>
Esophageal adenocarcinoma				
Case/control (1330/4705)				
All studies	15	0.131	29.9	0.59 (0.51, 0.68)
Population-based studies	8	0.106	40.9	0.62 (0.52, 0.73)
Clinical-based studies	7	0.319	14.5	0.53 (0.40, 0.68)
Eastern study	1	-	-	-
Western studies	14	0.128	31	0.60 (0.52, 0.68)
Studies with matched controls	10	0.139	33.6	0.62 (0.53, 0.72)
Studies without matched controls	5	0.333	12.7	0.49 (0.36, 0.66)
<i>Hp</i> <sup>+</sup> definition as <i>HpSe</i> <sup>+</sup>	8	0.299	16.6	0.55 (0.45, 0.66)
<i>Hp</i> <sup>+</sup> definition as <i>His</i> <sup>+</sup>	4	0.334	11.7	0.46 (0.33, 0.64)
Adjusted results	8	0.200	28.6	0.51 (0.40, 0.61)
<i>CagA</i> <sup>+</sup> vs <i>Hp</i> <sup>-</sup>	8	0.11	39.9	0.56 (0.45, 0.70)
Eastern study	0	-	-	-
Western studies	8	0.11	39.9	0.56 (0.45, 0.70)

<sup>1</sup>*P* value for *Q* statistical in random effects model; <sup>2</sup>Higgins *I*<sup>2</sup> statistic for heterogeneity in random effects model; <sup>3</sup>The overall value synthesized by fixed effects model. *HpSe*<sup>+</sup>: Sero-positivity for antibodies to whole-cell; *CagA*<sup>+</sup>: Sero-positivity for antibodies to cytotoxin-associated gene-A.

tion (OR = 1.02, 95%CI: 0.80-1.24). In the sub-group analyses of “*Hp*<sup>+</sup> only definition as *HpSe*<sup>+</sup>”, “studies with matched controls”, “studies without matched controls” no significant correlation between *H. pylori* infection and ESCC risk was found.

As studies have indicated that individuals infected with *CagA*-positive *H. pylori* strains have a higher risk of developing peptic ulcers and gastric cancer compared to those harboring *CagA*-negative *H. pylori* strains<sup>[34,35]</sup>, the association between *CagA*<sup>+</sup> stains and ESCC was also evaluated (Figure 3A). The overall OR was 0.97 (95%CI: 0.76-1.24) and showed high heterogeneity among the studies (*I*<sup>2</sup> = 52.0%, *P*<sub>heterogeneity</sub> = 0.03). This high heterogeneity may be caused partly by regional or ethnic differences, as heterogeneity values may weaken during location subgroup analysis. Similarly, *CagA*<sup>+</sup> strains of infection may decrease the risk of ESCC in Eastern subjects (OR = 0.77, 95%CI: 0.65-0.92), but not in Western subjects (OR = 1.26, 95%CI: 0.97-1.63) or the overall population (OR = 0.90, 95%CI: 0.78-1.05).

### *H. pylori* infection reduced the risk of EAC

Forest plot analyses are shown in Figure 2B. The overall positive rate of *H. pylori* infection in EAC was 35.96% (479/1332), which was significantly lower than that in normal controls 44.00% (2070/4705; OR = 0.71,

95%CI: 0.63-0.81). Quantitative meta-analyses showed that, compared with the control group, the combined OR of EAC in the presence of *H. pylori* infection was 0.59 (95%CI: 0.51-0.68). Table 2 also shows the results of subgroup analyses. The summary OR (95%CI) for clinical-based and population-based studies were 0.62 (95%CI: 0.52-0.73) and 0.53 (95%CI: 0.40-0.68), respectively. In the sub-group analyses of “Western studies”, “studies with matched controls”, “studies without matched controls”, “*Hp*<sup>+</sup> definition as *HpSe*<sup>+</sup>” and “adjusted results”, *H. pylori* infection was also inversely associated with EAC risk. Furthermore, compared with the *Hp*-population, *CagA*<sup>+</sup> strains of *H. pylori* also played a protective role in EAC carcinogenesis (OR = 0.56, 95%CI: 0.45-0.70, Figure 3B). However, only one study focused on the Eastern population and the OR was 0.32 (95%CI: 0.10-1.02).

### Sensitivity analysis and publication bias

Sensitivity analysis was performed to assess the influence of each individual study on the pooled ORs by omitting a single study each time, and no substantial change in the corresponding pooled OR (data not shown) was observed. Begg's funnel plot and Egger's test were performed to assess publication bias. Begg's funnel plots were symmetrical (Figure 2), and the *P* values for ESCC

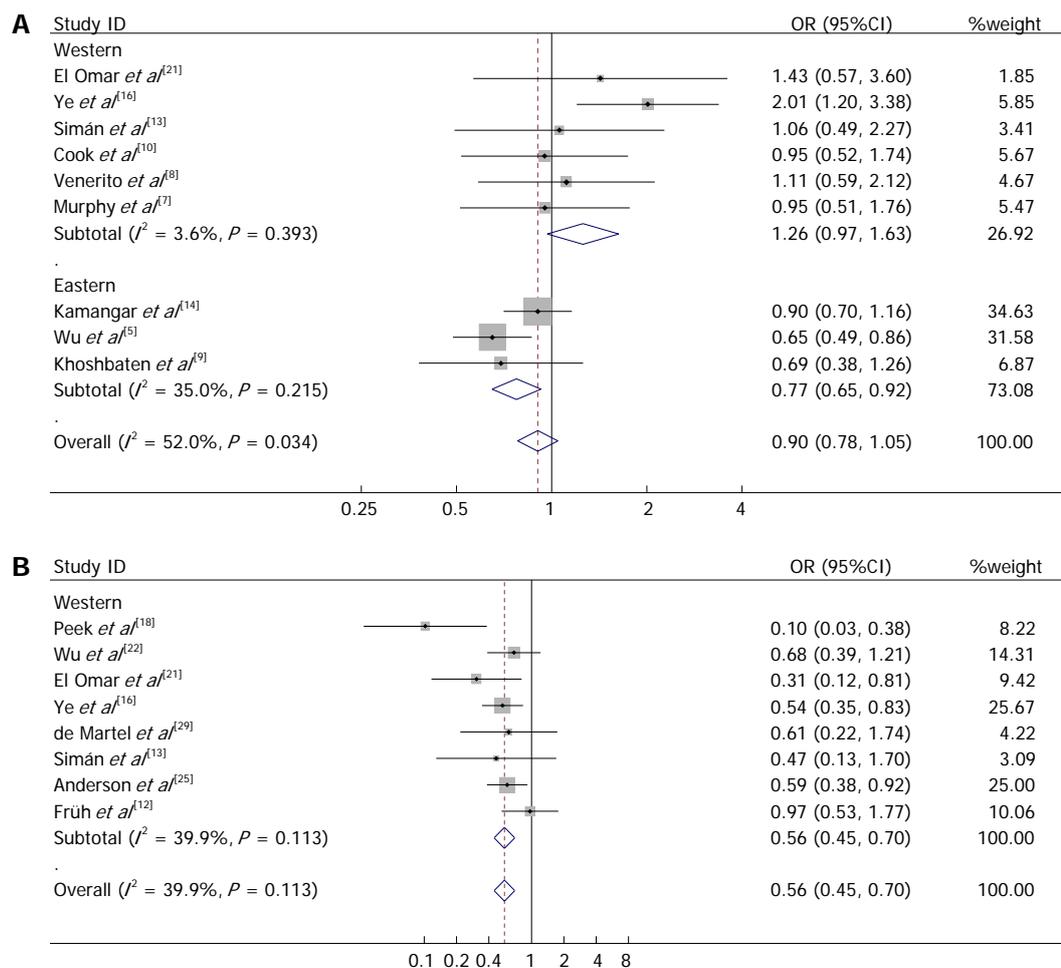


Figure 3 Meta-analysis with a fixed-effect model for the association between cytotoxin-associated gene-A-positive *Helicobacter pylori* infection and esophageal cancer. A: Esophageal squamous cell carcinoma (random-effect model); B: Esophageal adenocarcinoma (fixed-effect model).

and EAC were 0.753 and 0.621, respectively. The statistical results still did not show publication bias using Egger's test, and the *P* values for ESCC and EAC were 0.424 and 0.371, respectively. Therefore, there was no significant publication bias in the eligible studies.

## DISCUSSION

In the present study, we collected all available, published studies and performed a meta-analysis to examine the association between *H. pylori* infection and the risk of esophageal cancer. Twenty-seven studies were critically reviewed to clarify the controversial results from previous reports. Our meta-analysis showed that *H. pylori* infection significantly decreased the risk of EAC in Western populations. In terms of ESCC risk, no significant association was found when the Eastern and Western populations were pooled. In the stratified analysis of study location, no significant association between *H. pylori* infection and ESCC risk in Western subjects was found. However, we observed a significant association between *H. pylori* infection and decreased risk of ESCC in East Asian populations.

There are several explanations for this phenomenon.

There are fundamental differences in the carcinogenesis pathways between ESCC and EAC. Possible risk factors for ESCC include cigarette smoking, alcohol consumption, hot-temperature food, low intake of vegetables, salty food, pickled vegetables, nutrient deficiency, chronic mucosal irritation and a family history of cancer<sup>[36,37]</sup>, while EAC is closely related to Barrett's esophagus<sup>[38,39]</sup>. Genetic differences between ethnic groups may also induce diverse effects. For example, Umar *et al*<sup>[40]</sup> conducted a meta-analysis which showed that the PLCE1 polymorphism conferred significant risk for gastric and esophageal tumors in Asians (Chinese), but not in Caucasians. In Eastern populations, the incidence rates of EAC are generally higher in urban areas, where diet and lifestyle are similar to those in Western counties. Therefore, nutritional intake and lifestyle combined with *H. pylori* infection may have parallel effects in Eastern and Western populations<sup>[41,42]</sup>. In contrast, ESCC patients were mainly found in areas of Eastern developing countries, where nutrient absence and hot beverage intake are more universal than in Western populations. These different factors may influence the protective effect of *H. pylori* infection. Genetic factors, tumor biological characteristics and their complicated interactions with environmental factors may modulate risk

in ESCC.

Our study also showed that *H. pylori* infection is a strong protective factor against EAC, which is highly consistent with previous reports<sup>[4,43]</sup>. The underlying mechanism whereby *H. pylori* infection protects the esophagus has not been fully elucidated. *H. pylori* infection-related gastritis may result in lower gastric acid secretion<sup>[44]</sup>. Hypoacidity induced by atrophic gastritis has been proffered as one reason for this inverse association with EC. *H. pylori* infection reduced ghrelin synthesis in infected persons, which induced early satiety thereby preventing obesity and rapid gastric emptying, thus reducing the likelihood of gastroesophageal reflux, which may explain this protective effect<sup>[45]</sup>.

Two other meta-analyses have summarized the relationship between *H. pylori* infection and EC risk<sup>[4,43]</sup>. The advantages of our meta-analysis are as follows: Compared with the previous two meta-analyses, the present study was much larger, with more than twice as many cancer cases as the earlier studies. In addition, several subgroup analyses were conducted to identify potential sources of heterogeneity. Secondly, according to our selection criteria, all the studies in our meta-analysis had acceptable quality and the cases and controls were collated from all included studies, which significantly increased the statistical power. Thirdly, our study suggested that *H. pylori* infection decreased the risk of ESCC. This study should be repeated which could be beneficial in detecting novel mechanisms to reduce the risk of EC. We also found that our study had several limitations. Heterogeneity for the ORs in ESCC was observed among the studies. This heterogeneity may be due to various factors, such as diversity in the population characteristics, differences in the number of cases and controls, *H. pylori* detection methods and study design. However, heterogeneity was eliminated in the Western population after stratifying by ethnicity. The variables used to adjust these values were not consistent across the studies, which may limit the reliability of the data. Too few studies were identified to allow for subgroup analysis by covariates. Subgroup analyses regarding other confounding factors such as age and gender were conducted in the present study, but did not reduce the heterogeneity in the Eastern population. Only one study focused on the relationship between *H. pylori* infection and EAC risk in Eastern subjects (OR = 0.32, 95%CI: 0.10-1.02) which was not statistically significant ( $P = 0.05$ ). Further studies are required to confirm the protective role of *H. pylori*.

In conclusion, despite these limitations, our meta-analysis indicated that *H. pylori* infection may contribute to the decreased risk of EAC in the overall population and of ESCC in the Eastern population. To confirm our findings, further well-designed studies with large sample size and standardized laboratory methods in diverse ethnic populations should be performed to validate this association. The potential molecular mechanism of these protective effects should also be clarified to reduce the high morbidity caused by this malignancy.

## COMMENTS

### Background

Esophageal cancer is one of the most deadly malignancies. Many studies have explored the association between *Helicobacter pylori* (*H. pylori*) infection and esophageal cancer risk. However, the results were inconclusive and even controversial. Therefore, it is necessary to perform a meta-analysis in order to obtain a more precise evaluation of the relationship between *H. pylori* infection and esophageal cancer risk.

### Research frontiers

*H. pylori* has been identified as a pathogen in gastric cancers. To date, there have been many case-control studies on the association between *H. pylori* infection and esophageal cancer risk, but few meta-analyses have been conducted on this topic.

### Innovations and breakthroughs

This meta-analysis indicated that *H. pylori* infection might play a protective in esophageal squamous cell carcinoma (ESCC) risk in Eastern populations and in esophageal adenocarcinoma (EAC) risk in the overall population. Further studies are required to confirm these findings.

### Applications

*H. pylori* infection is inversely associated with ESCC risk in Eastern populations and with EAC risk in the overall population. This meta-analysis provided a structured and systematic integration of information on the etiology of esophageal cancer, and the results may provide valuable information for researchers and clinicians.

### Terminology

In cytotoxin-associated gene-A (CagA) positive strains of *H. pylori* the genome contains the cag pathogenicity island. This island includes approximately 31 putative genes, including CagA the gene that encodes the CagA protein strains that translocate the CagA protein into host cells and are significantly more likely to cause gastric cancer and other gastric diseases than CagA-negative strains.

### Peer review

These researchers performed a meta-analysis to clarify the association between *H. pylori* infection and development of esophageal carcinoma. The results are very important and create other questions in mind that lead to further studies.

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## Removal of an embedded "covered" biliary stent by the "stent-in-stent" technique

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metal stent; Endoscopic retrograde cholangiopancreatography; Biliary stricture; Jaundice

**Core tip:** Inability to retrieve a fully covered biliary self-expanding metal stent (SEMS) due to potential fixation of the stent to the duct wall can be addressed by the insertion of a second SEMS within the existing one, which facilitates the release of the initial SEMS.

Menon S. Removal of an embedded "covered" biliary stent by the "stent-in-stent" technique. *World J Gastroenterol* 2013; 19(36): 6108-6109 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6108.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6108>

### Abstract

A 46-year-old man was admitted with obstructive jaundice and cross-sectional imaging with computed tomography suggested distal biliary obstruction. A distal common bile duct stricture was found at endoscopic retrograde cholangiopancreatography (ERCP) and cytology was benign. A 6 cm fully covered self-expanding metal stent (SEMS) was inserted across the stricture to optimize biliary drainage. However, the SEMS could not be removed at repeat ERCP a few months later. A further fully covered SEMS was inserted within the existing stent to enable extraction and both stents were retrieved successfully a few weeks later. Fully covered biliary (SEMS) are used to treat benign biliary strictures. This is the first reported case of inability to remove a fully-covered biliary SEMS. Possible reasons for this include tissue hyperplasia and consequent overgrowth into the stent proximally, or chemical or mechanical damage to the polymer covering of the stent. Application of the stent-in-stent technique allowed successful retrieval of the initial stent.

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**Key words:** Self-expanding metal stent; Self expanding

### INTRODUCTION

The endoscopic management of benign biliary strictures has been transformed by the advent of removable fully covered metal stents. We report a case in which a fully covered biliary stent could not be removed endoscopically initially.

### CASE REPORT

A 46-year-old man was admitted with abdominal pain and obstructive jaundice [Bilirubin 14 mg/dL, Alanine aminotransferase (ALT) 123 IU/L, Alkaline phosphatase (ALP) 640 IU/L and a raised Amylase 350 U/L]. Cross-sectional imaging with computed tomography confirmed an enlarged and markedly calcified head of pancreas (Figure 1). Endoscopic retrograde cholangiopancreatography (ERCP) revealed a 2.5 cm distal common bile duct (CBD) stricture with proximal biliary dilatation. A 6 cm fully covered self-expanding metal stent (SEMS) (Wallflex, Boston Scientific, Boston, MA, United States) was inserted across the stricture. Cytology from the stricture was



Figure 1 Cholangiogram outlining a distal biliary stricture (arrow).

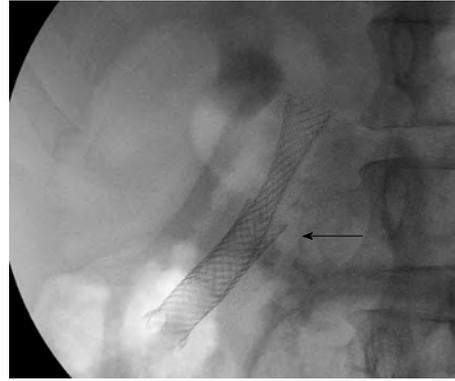


Figure 2 Second covered stent deployed over existing stent (arrow).

benign. At repeat ERCP 5 mo later, the SEMS could not be removed endoscopically with suggestion of the stent having embedded into the bile duct wall. Mild residual stricturing was noted at the apex of the SEMS. Further dilatation of the stricture was carried out to 10 mm and an in-stent dilatation was carried out to 12 mm to release the stent from the duct wall without success. At further ERCP in the next few weeks, a second 6 cm fully covered SEMS was inserted across the existing stent (Figure 2). Six weeks later, both stents were removed easily. Cholangiography suggested complete resolution of the original stricture. Examination of the initially inserted stent suggested possible damage to the silicone polymer stent covering (Figure 3) and is subject to further investigation.

## DISCUSSION

Embedded SEMS in the esophagus can be removed by inserting a fully covered SEMS within the SEMS, which causes pressure necrosis of tissue across the uncovered portion of the embedded SEMS, thereby facilitating its removal<sup>[1]</sup>. This technique has been used previously for removing uncovered biliary SEMS<sup>[2,3]</sup>.

Fully covered biliary (SEMS) are used widely to treat benign biliary strictures. This is the first reported case of inability to remove a fully-covered biliary SEMS. Possible reasons for this include tissue hyperplasia and consequent overgrowth into the stent proximally, or chemical or mechanical damage to the polymer covering of the stent. Application of the stent-in-stent technique allowed successful retrieval of the initial stent.

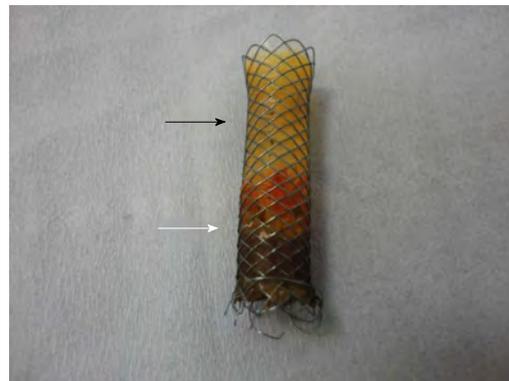


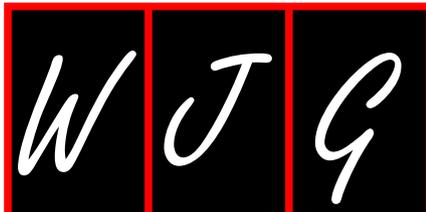
Figure 3 Covered stent after extraction. The white arrow indicates its duodenal end. There is evidence of damage to the polymer covering of the stent (black arrow).

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## Magnetic resonance venography and liver transplant complications

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in a patient with hepatic venous stenosis following liver transplantation. Initial venography failed to outline the stenoses and thus MRV using a blood pool contrast agent was utilised in order to delineate the anatomy and plan a therapeutic endovascular procedure.

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**Key words:** Magnetic resonance venography; Blood pool; Magnetic resonance angiography; Nephrogenic systemic fibrosis; Liver transplant; Gadofosveset trisodium

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### Abstract

Hepatic vein stenosis is a rare but serious complication following liver transplantation. Multiple modalities can be utilized to image the hepatic vasculature. Magnetic resonance venography (MRV) provides certain advantages over ultrasound, computed tomography angiography and digital subtraction venography. MRV utilizes the same imaging principles of magnetic resonance angiography in order to image the venous system. Blood pool contrast agents, specifically gadofosveset trisodium, allow for steady state imaging up to 1 h following injection, with improved visualisation of vital venous structures by utilising delayed steady state imaging. Additionally, the inherent physics properties of magnetic resonance imaging also provide excellent soft tissue detail and thus help define the extent of complications that often plague the post-liver transplant patient. This case report describes the use of gadofosveset trisodium

### INTRODUCTION

The inherent physics principles of magnetic resonance imaging (MRI) make it extremely sensitive to blood flow. MRI investigations of blood flow have been targeted at investigation of the arterial system; the same principles can be applied to the venous system for the diagnosis and planning of therapy targeted at improving, re-establishing or decreasing venous flow, depending on the underlying diagnosis. Digital subtraction venography (DSV) is regarded as the most accurate method for diagnosis of venous thrombosis as well as venous stenosis<sup>[1]</sup>. However, DSV has associated morbidity related to radiation dose, intravenous contrast injection and venous puncture.

Magnetic resonance angiography (MRA) uses specific acquisition sequences to highlight blood flow and is widely used to assist in the management of patients with vascular disease<sup>[2]</sup>.

Magnetic resonance venography (MRV) utilizes the

same imaging principles as MRA in order to image the venous system. Blood pool contrast agents such as gadofosveset trisodium (Ablavar, Lantheus, N Bellerica, Mass, United States) allow improved first pass imaging due to the increased relaxivity of the contrast agent<sup>[3]</sup>. The important clinical property that makes it ideal for venous imaging is the longer intravascular dwell time, allowing for steady state acquisitions up to 1 h following injection. Delayed, steady state imaging facilitates improved visualization of vital venous structures with a small decay in signal.

This case report presents a novel application of a blood pool contrast agent in planning an intervention in a liver transplant patient with perianastomotic hepatic vein stenosis.

## CASE REPORT

A 47-year-old female was referred to the interventional radiology department with refractory ascites following a live donor liver transplant 10 mo previously for papillary cholangiocarcinoma.

A transjugular cavogram demonstrated an inferior vena cava (IVC) stenosis, however we were unable to reflux contrast into the transplant hepatic veins (Figure 1). A decision was made to abandon the procedure and perform further imaging in order to demonstrate and delineate the hepatic venous anastomosis. An US study was originally performed but the result was deemed insufficient to delineate the anatomy prior to further intervention.

A contrast enhanced MR venogram (GE Healthcare 1.5T, Giles, United Kingdom) was performed using Gadofosveset Trisodium (Ablavar, Lantheus, N Bellerica, Mass, United States). Spoiled gradient echo sequences were obtained using first pass imaging (Coronal acquisition, Flip angle: 30°, FoV: 40 cm, section thickness 2.8 mm, frequency coding: 320, phase coding: 192, NEX: 0.75, K-space filling: centric, spatial resolution: 1.25/2.1) at 25 s and steady state imaging (Coronal acquisition, Flip angle: 20°, FoV: 40 cm, section thickness 2.8 mm, frequency coding: 320, phase coding: 320, NEX: 1, K-space filling: centric, spatial resolution: 1.25/1.25) at 2 min and 5 min after injection of 10 mL Gadofosveset Trisodium (1 mL/s) followed by a 30 mL saline flush (1 mL/s).

The first pass images demonstrated a patent hepatic arterial anastomosis, with no focal arterial pathology seen. The 5-min steady state imaging demonstrated a patent hepatic venous stenosis with a significant perianastomotic stenosis (Figure 2A) and an IVC stenosis (Figure 2B). No hepatic or portal venous thrombosis was seen.

A combined transhepatic and transjugular hepatic venoplasty was performed following review of the imaging. Initially, an ultrasound guided transhepatic puncture of the right hepatic vein was performed and a guidewire inserted across the perianastomotic stenosis into the right atrium (Figure 3). In order to obtain through and through access to the hepatic venous and IVC stenosis, a



Figure 1 Digital subtraction venogram *via* a pigtail catheter placed in the infrahepatic inferior vena cava demonstrating inferior vena cava stenosis (arrow) and no reflux of contrast into the transplant hepatic veins.

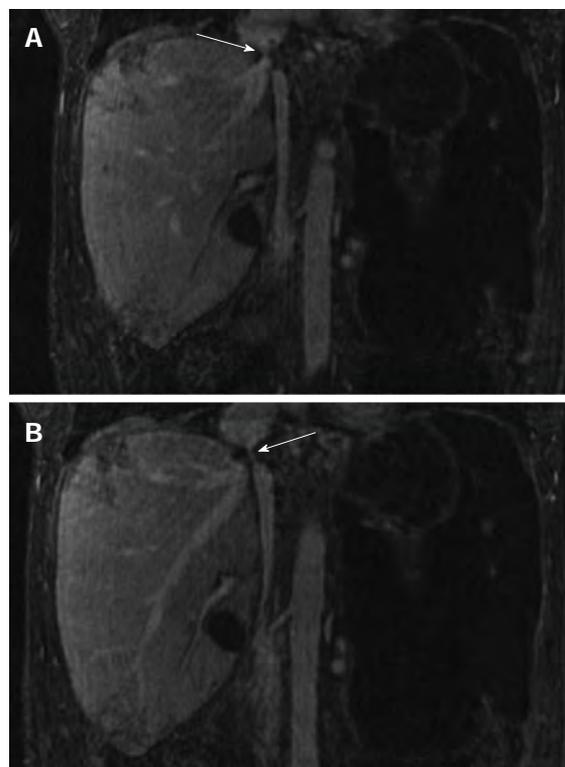


Figure 2 Five-minute steady state imaging. A: Coronal T1 weighted ultrafast gradient echo steady state magnetic resonance imaging image obtained 5 min following administration of Gadofosveset trisodium demonstrates inferior vena cava stenosis (white arrow); B: Coronal T1 weighted ultrafast gradient echo steady state magnetic resonance imaging image obtained 5 min following administration of gadofosveset trisodium demonstrating patent, but stenosed transplant hepatic vein (white arrow).

loop snare was introduced from a jugular venous access site and the guidewire was snared and retracted through the jugular venous access site (Figure 4). The stenoses were dilated using a high-pressure balloon (Atlas, Bard Medical, Tempe, AZ, United States), with satisfactory angiographic appearances post venoplasty (Figure 5). Both stenoses were treated concurrently due to their close proximity. The hepatic venous/right atrial gradient decreased from 15 to 8 mmHg following the procedure;

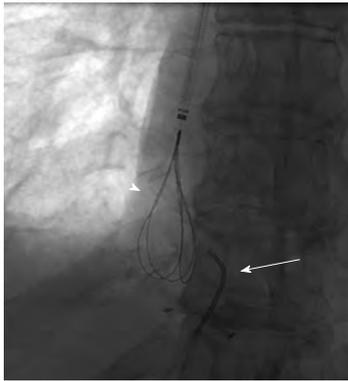


Figure 3 Fluoroscopic image demonstrating a loop snare inserted through the internal jugular vein access (arrowhead), and an angled catheter (arrow) through the right hepatic vein. Access to the right hepatic vein was achieved *via* percutaneous ultrasound guided puncture.

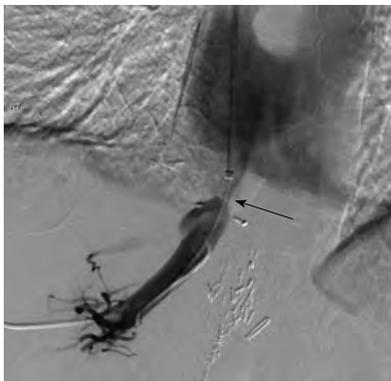


Figure 4 Digital subtraction venogram demonstrating through and through access across the hepatic venous stenosis (arrow).

due to the close proximity of the hepatic venous and IVC stenoses, a separate gradient across the IVC stenosis was not felt to be practical given a second catheter would need to be placed in the infra-hepatic IVC, in order to maintain the hepatic venous access. The patient was monitored post-procedure for four hours as per departmental protocol and discharged with no peri-procedural complications.

The patient was reviewed three months post treatment at our transplant clinic, where the ascites had completely resolved and she had resumed her normal daily activities. Doppler ultrasound demonstrated normal flow in the hepatic vein with no evidence of recurrent stenosis. A follow-up MRI was performed 1-year post venoplasty as part of annual surveillance to exclude tumor recurrence. The MRI demonstrated no evidence of ascites (Figure 6), which together with the patient's normal liver function parameters and clinical examination, confirmed a satisfactory result. Follow-up venography was not felt to be necessary given the clinical response.

## DISCUSSION

This technical report demonstrates the benefit of steady-

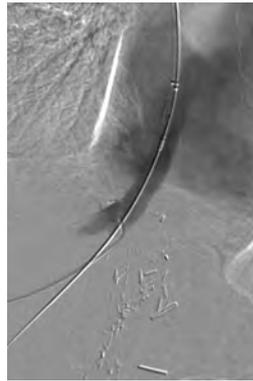


Figure 5 Completion venoplasty demonstrating satisfactory post-venoplasty appearances of the hepatic vein.

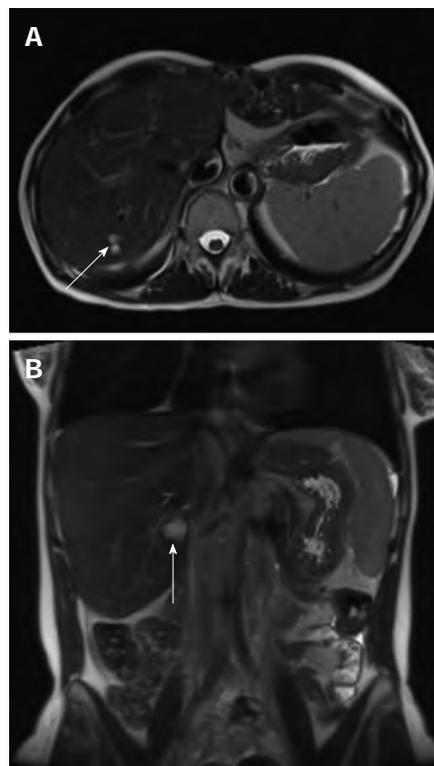


Figure 6 Follow up magnetic resonance imaging axial (A) and coronal (B) balanced gradient echo sequences (True FISP; Siemens Medical, Erlangen, Germany) through the liver performed as part of routine annual tumor surveillance at 1 year following venoplasty of the hepatic vein and inferior vena cava stenosis demonstrated normal appearance of the liver apart from simple parenchymal cysts (arrow). No ascites was seen.

state imaging using a blood pool contrast agent, Gadofosveset Trisodium. MRI provides exquisite soft tissue contrast and allows for the delineation of liver transplant anatomy either prior to embarking on a therapeutic intervention or if conventional angiography is unsuccessful as in this case. Cannulation of the stenosed hepatic vein proved technically challenging and without adequate reflux of contrast into the hepatic veins, cross-sectional imaging was required in order to plan a safe combined transhepatic and transjugular approach.

Hepatic vein stenosis is a rare but serious complication after liver transplantation<sup>[4]</sup>. It has been reported to occur from 1% to 6% of the cases. It can occur both acutely or in a delayed fashion. Acute hepatic vein stenosis is thought to be due to surgical technique whereas delayed stenosis is caused by intimal hyperplasia or perianastomotic fibrosis<sup>[4]</sup>. Clinical signs of hepatic vein stenosis include hepatic dysfunction, liver engorgement, ascites, and occasionally variceal bleeding. If the stenosis occurs at the level of the suprahepatic IVC as in this case, renal dysfunction and lower extremity edema may occur.

Gadofosveset Trisodium (Ablavar, Lantheus, N Bellerica, Mass, United States) reversibly binds to serum albumin, providing significantly higher relaxivity and prolonged intravascular enhancement compared to existing extracellular MR contrast agents<sup>[2]</sup>. The optimal dose is 0.03 mmol/kg, however in our experience greater than 10mL of contrast is not required in the majority of patients. The safety and efficacy of the contrast medium has been studied using a variety of clinical scenarios including aorto-iliac occlusive disease and pedal arterial disease which demonstrated the contrast agent to improve the rate of interpretability and improved diagnostic confidence<sup>[2]</sup>.

The significantly longer intravascular time allows equilibrium imaging due to a prolongation of the T1 relaxivity, despite the inevitable dilution of the injected contrast medium after first pass imaging<sup>[5]</sup>. Prolonged T1 reduction thus allows diagnostic imaging of the vascular tree 45-60 min after injection of contrast. The increased imaging window can be used to acquire images with much higher spatial resolution without a significant loss of vessel to background contrast.

A rapid injection rate is not required. A saline chaser of at least 30 mL at a rate similar to the injection rate of the contrast medium ensures the full dose of contrast medium enters the central circulation without affecting the signal to noise ratio (SNR)<sup>[6]</sup>.

The use of Gadofosveset Trisodium for problem solving in liver transplant patients, to our knowledge has not been described. The same parameters utilized for our traditional abdominal first pass and steady state imaging were utilized for image acquisition and provided the interventional radiology team with the anatomical detail

required to perform the necessary venoplasty.

The favourable safety profile and the low dose required to obtain diagnostic images (0.03 mmol/kg) makes this agent preferable as to date, there have been no reported cases of NSF associated with gadofosveset trisodium<sup>[2,7]</sup>.

In conclusion, MRV of transplant livers using a blood pool contrast agent is an accurate, noninvasive method of evaluating the vascular anastomoses (arterial, hepatic and portal venous systems). The longer intravascular dwell time of Gadofosveset Trisodium allows imaging with little loss of SNR. MRV with a blood pool contrast agent is a robust MRI technique, which eliminates contrast bolus timing, provides good temporal and spatial resolution and allows for dynamic image acquisition whilst compensating for differences in patient blood flow dynamics.

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## Large symptomatic gastric diverticula: Two case reports and a brief review of literature

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### Abstract

Gastric diverticula are rare and uncommon conditions. Most gastric diverticula are asymptomatic. When symptoms arise, they are most commonly upper abdominal pain, nausea and emesis, while dyspepsia and vomiting are less common. Occasionally, patients with gastric diverticula can have dramatic presentations related to massive bleeding or perforation. The diagnosis may be difficult, as symptoms can be caused by more common gastrointestinal pathologies and only aggravated by diverticula. The appropriate management of diverticula depends mainly on the symptom pattern and as well as diverticulum size. There is no specific therapeutic strategy for an asymptomatic diverticulum. Although some authors support conservative therapy with antacids, this provides only temporary symptom relief since it is

not able to resolve the underlying pathology. Surgical resection is the mainstay of treatment when the diverticulum is large, symptomatic or complicated by bleeding, perforation or malignancy, with over two-thirds of patients remaining symptom-free after surgery, while laparoscopic resection, combined with intraoperative endoscopy, is a safe and feasible approach with excellent outcomes. Here, we present two cases of uncommon large symptomatic gastric diverticula with a discussion of the cornerstones in management and report a minimally invasive solution, with a brief review of the literature.

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**Key words:** Gastric diverticulum; Laparoscopic gastric diverticulectomy; Abdominal pain; Dysphagia; Gastric; Diverticulum

**Core tip:** Gastric diverticula are infrequent anatomic abnormalities and are usually asymptomatic. They present with variable symptoms. Although most symptomatic patients are diagnosed during evaluation of vague epigastric discomfort, severe complications, including perforation and hemorrhage, may occur. We report a successful laparoscopic approach as a minimally invasive solution to a symptomatic gastric diverticula with a brief literature review on this rare condition. Knowledge of the pitfalls in diagnosis and treatment of a gastric diverticulum are essential for successful and complete relief of symptoms.

Marano L, Reda G, Porfidia R, Grassia M, Petrillo M, Esposito G, Torelli F, Cosenza A, Izzo G, Di Martino N. Large symptomatic gastric diverticula: Two case reports and a brief review of literature. *World J Gastroenterol* 2013; 19(36): 6114-6117 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6114.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6114>

## INTRODUCTION

A gastric diverticulum is a pouch protruding from the gastric wall and has similar characteristics to duodenal, jejunal and colonic diverticula<sup>[1]</sup>. Generally it is a very rare and uncommon condition with a prevalence of 0.04% in contrast radiographs and 0.01%-0.11% in upper gastrointestinal endoscopies<sup>[2,3]</sup>. It occurs equally in men and women, typically in the fifth and sixth decades. Although most patients are asymptomatic, occasionally abdominal symptoms occur, including vague pain, epigastric fullness, bleeding or perforation<sup>[4-6]</sup>. Gastric diverticula are usually 1-3 cm in diameter and can be divided into true diverticula comprising all gastrointestinal layers and pseudodiverticula which are often found in the antrum<sup>[7,8]</sup>. We present two cases of uncommon large symptomatic gastric diverticula with discussion of the cornerstones in management, and report a minimally invasive solution, with a brief review of literature.

## CASE REPORT

Two 51-year-old and 49-year-old women were referred to our Department for evaluation of symptoms of epigastralgia and upper abdomen tenderness, respectively. They denied weight loss, hematemesis and melena. The patients did not complain of vomiting or any abnormal bowel function. Abdominal examinations revealed no masses or other abnormalities. A barium esophagogastric study was performed, which showed an image of a protruding pouch in the upper gastric region (Figure 1). To confirm the diagnosis, an upper endoscopy was conducted, demonstrating diverticula directed posteriorly off the fundus of the stomach that measured 5 cm and 8 cm in diameter, respectively (Figure 2). The diverticula had a narrow neck but no ulcer was identified. Functional examination with esophageal stationary manometry and 24-h esophagogastric pH-multichannel intraluminal impedance monitoring showed normal values. The electrogastrography monitoring showed an increase in bradyarrhythmic gastric activity in both patients. Patients were presented for surgery and the operations were performed laparoscopically. The interventions were substantially the same for both patients. Because inspection of the stomach did not show a diverticulum at the anterior surface, the bursa omentalis was opened by dividing the gastrocolic omentum. Under endoscopic control, the diverticula were resected at the neck with EndoGIA (Universal, US Surgical Corporation, Norwalk, CO, United States) and then successfully retrieved with a laparoscopic pouch (Figure 3). Then, the staple lines were carefully examined and tested by submerging insufflated stomach under sterile irrigation to observe bubbles and there was no evidence of a leak. Histology confirmed a gastric diverticula of 5 cm and 8 cm, respectively, with normal mucosa in both cases. The procedures were uneventful. Patients were placed on a regular diet on postoperative day 3 and discharged 5 d later. Patients did not show any complications after surgery. On their first 6-mo follow-up visit, neither patient complained of any

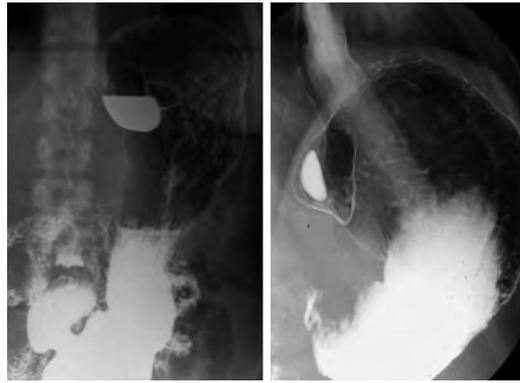


Figure 1 A barium study reveals diverticula directed posteriorly off the fundus of the stomach that measured 5 cm and 8 cm in diameter in 51-year-old and 49-year-old women, respectively.

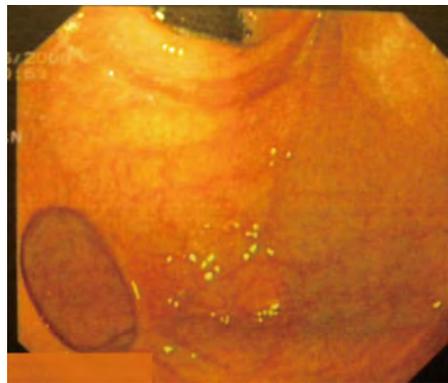


Figure 2 Endoscopic view of gastric diverticulum with a narrow neck.

symptoms, while normal radiographic and endoscopic examinations, and regular values of esophageal manometry and 24-h esophagogastric pH-multichannel intraluminal impedance were found.

## DISCUSSION

Moebius in 1661 and later Roax in 1774 first described gastric diverticula<sup>[9]</sup>. The literature suggests that most symptomatic diverticula are found in patients between 20 and 60 years of age<sup>[10-12]</sup>, while a unique study showed that only 4% of gastric diverticula occurred in patients younger than 20 years<sup>[11]</sup> and no differences in incidence between sexes have been described<sup>[4]</sup>. Two types of gastric diverticula are recognized according to Akerlund<sup>[13]</sup> and Schmidt *et al.*<sup>[14]</sup>: congenital (true) and acquired (false) diverticula, with congenital types being more common<sup>[2,15-17]</sup>. True diverticula have all layers of the gastric wall, and it is believed that these congenital diverticula occur as a result of splitting of the longitudinal muscular fibers at the cardia level, leaving only circular muscle fibers in the gastric wall and creating a weakening to allow a diverticulum to form during the fetal period. This hypothesis is supported by Reich<sup>[18]</sup> who reported fetal gastric diverticula and by Lewis, which described gastrointestinal diverticula in embryos in 1908<sup>[19]</sup>. False diverticula, also classified as pulsation and traction diverticula, do not car-

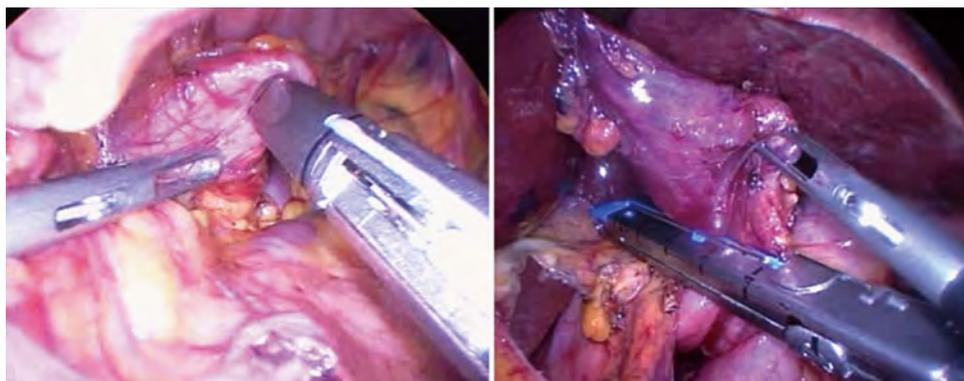


Figure 3 Exposure of the neck of the diverticulum for preparation of diverticulectomy and resection at the neck with a linear stapler.

ry all layers of the gastric wall<sup>[20]</sup>. Pulsation diverticula are those arising from increased intraluminal pressure, such as chronic coughing, obesity and pregnancy. Traction diverticula arise from perigastric adhesions from concurrent diseases, such as peptic ulcer disease, pancreatitis, malignancy, gastroesophageal reflux and cholecystitis<sup>[11,12]</sup>. Gastric traction diverticula have been reported after surgical procedures on the stomach, including Roux-en-Y gastric bypass<sup>[12,21,22]</sup>. Even if gastric diverticula can arise virtually anywhere along stomach, most congenital diverticula (70%) are typically located on the posterior wall of the stomach just below the gastroesophageal junction<sup>[2]</sup>, while acquired diverticula are usually situated near the gastric antrum. Typical diverticula are 1-3 cm in diameter but large diverticula as demonstrated above can occur<sup>[2,15]</sup>.

Most gastric diverticula are asymptomatic<sup>[22]</sup>, however when symptoms arise, depending on the size of the diverticular neck, they are most commonly upper abdominal pain, nausea, and emesis, and are described in 18%-30% of cases<sup>[2,21]</sup>. Wide-neck diverticula often go unnoticed perhaps because food and digestive juices are less likely to become trapped. It has been suggested that food retention with subsequent distension of the gastric diverticulum may cause pain<sup>[23,24]</sup>. Dyspepsia and vomiting are less common<sup>[21,25]</sup>. Occasionally, patients with gastric diverticula can have dramatic presentations related to massive bleeding or perforation<sup>[26]</sup> due to food retention with subsequent release of gastric juices within the mucosal sac, causing diverticulitis and possibly ulceration or hemorrhage. There are two reports of invasion with adenocarcinoma<sup>[27,28]</sup>. The diagnosis may be difficult, as complaints can be caused by more common gastrointestinal pathologies and only be aggravated by diverticula. Methods of detection can fail, therefore, a combined approach should be used<sup>[8]</sup>. The presence of a mucosal sac can be confirmed with upper gastrointestinal contrast studies and endoscopies. These are the most reliable diagnostic tests but reports in the literature confirm that they can still miss the lesion if it has a narrow neck that precludes entry of the contrast or scope, giving false negative results<sup>[2,11,29]</sup>. In a large review, Palmer<sup>[11]</sup> reported that 5% of gastric diverticula are missed during upper gastrointestinal investigation. Other reports recommend the use of upper endoscopy<sup>[22,30]</sup> for diagnosis,

as this modality easily confirms the location and size of the diverticulum and provides the opportunity to biopsy any concurrent pathology. This diagnostic tool can rule out associated pathology and may be able to reproduce symptoms with distention of the diverticulum, indicating which patients would benefit from resection<sup>[15,22]</sup>. Computerized tomography scans are also used to diagnose diverticula; however, these have also mistaken diverticula for adrenal masses<sup>[31]</sup>.

Appropriate management of a diverticulum depends mainly on the symptoms as well as on the diverticulum size. There is no specific therapeutic strategy for an asymptomatic diverticulum<sup>[5,18]</sup>. However, routine surveillance with a periodic physical examination is recommended, given the potential onset of complications<sup>[20]</sup>. Diverticula exceeding 4 cm are more prone to produce complications and tend to respond less favorably to medications. Although some authors support conservative therapy with antacids, this provides only temporary symptom relief and is not able to resolve the underlying pathology<sup>[20]</sup>. A necessity for successful treatment with complete relief of symptoms is the association of the symptoms with the diverticulum. Palmer<sup>[11]</sup> found that in 30 of 49 symptomatic patients with a gastric diverticulum, symptoms were attributable to other gastrointestinal diseases, and 6 of 9 patients with symptoms caused by a gastric diverticulum who underwent open surgery showed excellent outcomes. Resection of gastric diverticula in all patients will lead to unsatisfactory results<sup>[22]</sup>. Surgical resection is the mainstay of treatment when the diverticulum is large, symptomatic or complicated by bleeding, perforation or malignancy, with over two-thirds of patients remaining symptom-free after surgery<sup>[11]</sup>. Several surgical approaches have been described including invagination of the diverticulum as well as partial gastrectomy<sup>[32,33]</sup>, however, since the first successful laparoscopic resection of a gastric diverticulum in the late 1990s, this approach is now considered safe and feasible<sup>[34]</sup>. The most favorable approach providing better exposure is by placing a, right upper quadrant port, and 2 left upper quadrant ports. Laparoscopic dissection has been performed by either releasing the gastrocolic ligament, thus allowing exposure of the superior posterior wall of the stomach<sup>[5,34-36]</sup>. Simple diverticulum resection

with a laparoscopic cutting stapler has been reported to be successful<sup>[37]</sup>. In some cases the surgical approach can be challenging because the diverticulum is often collapsed and hidden in the splenic bed. Sometimes, a resection of the wrong part of the stomach has also been described<sup>[22]</sup>. For this reason the surgical procedure should be combined with intraoperative endoscopy to find an elusive diverticula by stretching the diverticular sac<sup>[4,34-36]</sup>.

In conclusion, laparoscopic resection is a safe and feasible surgical approach with excellent outcomes and is strongly indicated for symptomatic gastric diverticula.

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## Isolated right posterior bile duct injury following cholecystectomy: Report of two cases

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### Abstract

Anatomic variations of the right biliary system are one of the most common risk factors for sectoral bile duct injury (BDI) during cholecystectomy. Isolated right posterior BDI may in particular be a challenge for both diagnosis and management. Herein we describe two cases of isolated right posterior sectoral BDI that took place during laparoscopic cholecystectomy. Despite effective external biliary drainage from the liver hilum in both cases, there was a persistent biliary leak observed which was not visible on endoscopic retrograde

cholangiogram. Careful evaluation of images from both endoscopic and magnetic resonance cholangiograms revealed the diagnosis of an isolated right posterior sectoral BDI. These were treated with a delayed bisegmental (segments 6 and 7) liver resection and a Roux-en-Y hepaticojejunostomy respectively with good outcomes at 24 and 4 mo of follow-up. This paper discusses strategies for prevention of such injuries along with the diagnostic and therapeutic challenges it offers.

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**Key words:** Cholecystectomy; Bile duct injury; Sectoral bile duct; Hepaticojejunostomy; Liver resection

**Core tip:** Anatomic variations of the right biliary system are common but the low insertion of the right sectoral bile duct into the common hepatic duct (or the cystic duct) is rare. This is clinically important as places the patient at particular risk for its injury during cholecystectomy. Moreover, it can be very difficult to be diagnosed and managed properly. It often presents with a persistent biliary leak which is not visible on endoscopic retrograde cholangiogram. We describe two such cases, present the diagnostic imaging with two different treatment options and discuss preventive and management strategies. This can be of clinical value for both surgeons, gastroenterologists and endoscopists.

Wojcicki M, Patkowski W, Chmurowicz T, Bialek A, Wiechowska-Kozłowska A, Stankiewicz R, Milkiewicz P, Krawczyk M. Isolated right posterior bile duct injury following cholecystectomy: Report of two cases. *World J Gastroenterol* 2013; 19(36): 6118-6121 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6118.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6118>

## INTRODUCTION

Bile duct injury (BDI) is one of the most serious complications of both open and laparoscopic cholecystectomy. They often result from misidentification of anatomy by the operating surgeons and anatomic variations within the biliary tree<sup>[1]</sup>. Variations in biliary tree anatomy occur in about 25% of patients with aberrant right hepatic ducts being the most common. The low insertion of a right sectoral hepatic duct into the common hepatic duct or the cystic duct (Figure 1) increases the risk of injury during both open<sup>[2,3]</sup> and laparoscopic cholecystectomy<sup>[4,5]</sup>. Therefore, a detailed knowledge and awareness of all possible anatomical variants is crucial for surgeons to avoid complications.

Herein we describe the diagnosis and treatment of an isolated right posterior sectoral BDI sustained during laparoscopic cholecystectomy in two patients. We emphasize the preventive measures to be taken at surgery and discuss the diagnostic and therapeutic options.

## CASE REPORT

### Case 1

A 15-year-old female patient with a symptomatic gallstone disease underwent a laparoscopic cholecystectomy converted to an open procedure in November 2010 in a regional institution. The patient was discharged home on the 6<sup>th</sup> day post operation. She presented 5 d following discharge with abdominal pain and was found to have a subhepatic fluid collection on ultrasound scanning. At relaparotomy drainage of a biloma and abdominal lavage was performed. The patient was discharged home again 16 d following cholecystectomy with an abdominal drain left in place as it still produced around 150-200 mL of bile daily. Due to persistence of a biliary leak, endoscopic retrograde cholangiography (ERC) was performed 20 d after initial surgery. This showed no evidence of a leak and a consult of a hepatobiliary surgeon was requested. During careful evaluation of all the ERC images it became obvious that the right posterior (segments 6 and 7) sectoral bile duct was clearly missing (Figure 2). The diagnosis of an isolated Strasberg type C bile duct injury<sup>[6]</sup> was further confirmed by magnetic resonance cholangiography with maximum-intensity-projection using the slice-stacking technique and T2-weighted sequences. This showed a leak from the right posterior sectoral duct that was isolated from the remaining biliary system (Figure 3). Continued external drainage for a period of several weeks was recommended allowing time for the local inflammation to settle and for possible spontaneous closure of the leak or to serve as a bridge for a definitive repair. The leak persisted at around 150-250 mL/d at which stage definitive surgery was opted for. A Roux-en-Y hepaticojejunostomy to the damaged posterior sectoral duct was initially planned. However, an attempt to identify the damaged duct during surgery was unsuccessful. Therefore, a bisegmental (segments 6 and 7) hepatic resection

was performed in order to remove the affected parenchyma of the liver. The postoperative course was uneventful and the patient was discharged home 10 d after surgery. She remains well at present at 27 mo post cholecystectomy and 24 mo after definitive surgical treatment.

### Case 2

A 21-year-old female patient underwent cholecystectomy for acute cholecystitis in a regional hospital in September 2012. The procedure started laparoscopically but was converted to open surgery because of difficulties localizing anatomical structures. The patient was discharged 6 d following surgery. She was readmitted one week later with fever, abdominal pain and vomiting. Abdominal ultrasound confirmed a subhepatic fluid collection, which proved to be bilious in origin at percutaneous drainage. A drain was left in the subhepatic space and the biliary leak continued (200-300 mL/d) despite its absence on ERC performed. At this stage, 56 d post cholecystectomy, the patient was referred to the authors institute for further management. Following admission the patient was found to be in reasonably good health other than for the persistent biliary leak. A magnetic resonance cholangiogram was performed and this revealed a leak from a right posterior sectoral duct that had no communication with the remaining biliary tree (Strasberg type C injury)<sup>[6]</sup>. The ERC was performed once again and confirmed an intact intra and extrahepatic biliary tree with an absent right posterior duct. The patient was then offered surgical repair. During surgery, an open stump of the right posterior sectoral duct (confirmed by intraoperative cholangiography) measuring around 3 mm in diameter was identified. A Roux-en-Y hepaticojejunostomy was performed. The postoperative course was uneventful and the patient was discharged home 11 d after surgery. She remains well and asymptomatic at 4 mo of follow-up with undilated biliary tree on ultrasonography and normal values of serum bilirubin, alkaline phosphatase and transaminase levels.

## DISCUSSION

Dangerous anatomy, pathology and surgery are three main risk factors which may be responsible for BDI during open and laparoscopic cholecystectomy<sup>[7]</sup>. Incorrect interpretation of anatomy appears to be the most common cause of injury, particularly in isolated right sectoral BDI<sup>[4,5]</sup>. The most dangerous scenario includes the cystic duct located close to or joining the sectoral duct or the sectoral duct draining into the cystic duct or the gallbladder neck (Figure 1)<sup>[7,8]</sup>. This should be always kept in mind during surgery and anticipated until proven otherwise. In order to prevent the injury and identify any variant anatomy early, complete circumferential dissection of the gall bladder neck and the cystic duct should be performed before clip placement.

Anomalous drainage of the right posterior sectoral bile duct into the cystic duct or the common hepatic duct is seen in around 2%-5% of patients<sup>[4,5,9-11]</sup>. Its injury does

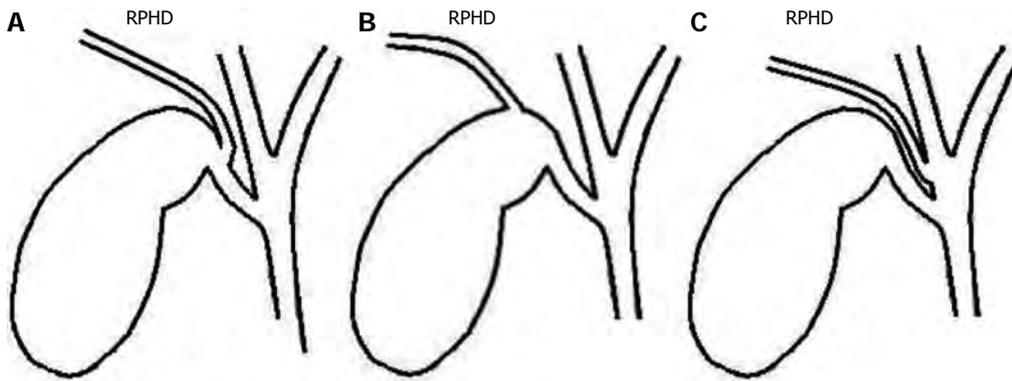


Figure 1 Dangerous anatomical variants of right posterior hepatic duct draining into the cystic duct (A), gall bladder neck (B) or the common hepatic duct (C). RPHD: Right posterior hepatic duct.



Figure 2 Endoscopic retrograde cholangiogram showing seemingly “normal” biliary tree with no evidence of a biliary leak. However, only the left hepatic duct (long arrow) and the right anterior hepatic duct (short arrow) are visible.

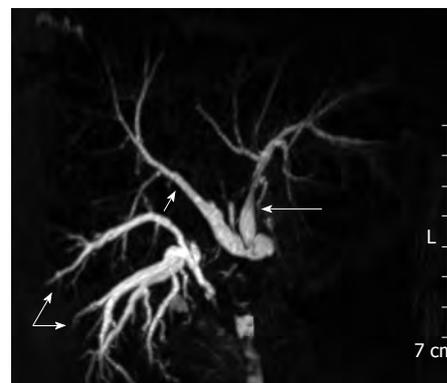


Figure 3 Magnetic resonance cholangiography with the use of T2-weighted sequences and maximum-intensity-projection imaging. The fluid-containing bile ducts as high signal-intensity structures; the right posterior hepatic ducts of segments 6 and 7 are shown (double arrow) with no connection to the right anterior duct (single short arrow) and the left hepatic duct (single long arrow); the abdominal drain placed in the subhepatic area and draining the bile externally is also visible in between the right anterior and posterior hepatic ducts.

not belong to the original classification of BDI described by Bismuth *et al*<sup>[12]</sup>. It is recognized though by Strasberg classification as the occlusion (type B) or a leak (type C) from a duct not in communication with the common bile duct<sup>[6]</sup>. The true incidence of Strasberg type B injury may be underestimated since some of them are likely to be asymptomatic and pass unnoticed leading to atrophy or segmental cirrhosis of part of the liver. While occlusion of the duct usually leads to cholestasis or recurrent episodes of cholangitis<sup>[3,13]</sup>, its transection presents mainly with signs of biliary peritonitis or external biliary leak *via* the abdominal drain<sup>[4,14-16]</sup>, which was the case in our patients.

Proper diagnosis and management of right sectoral BDI remains difficult. The results of ERC may be interpreted as “normal” with no biliary leaks visible (Figure 2). The leak may then be erroneously attributed to injury to a minor biliary radical in the gall bladder fossa (the ducts of Luschka) and delay the diagnosis. However, careful evaluation of the obtained ERC images may reveal that only the right anterior sector of the liver (segments 5 and 8) is visualized and the posterior (segments 6 and 7) missing<sup>[3,4]</sup> (Figure 2). Magnetic resonance cholangiography can further confirm the lack of communication between the injured duct and the common bile duct (Figure 3).

This makes endoscopic treatment impossible in such cases and is essential for treatment planning.

The treatment depends on the timing of recognition of the type of injury. External biliary drainage should allow prompt control of the leak, eliminate the risk of sepsis and serve as a bridge for definitive repair<sup>[4]</sup>. Total nonoperative management has recently been advocated as a way to obviate the need for surgery in up to 50% of patients<sup>[16]</sup>. If the leak persists beyond approximately 8 wk, treatment with Roux-en-Y hepaticojejunostomy is usually indicated<sup>[12,16,17]</sup>. However, the risk of late stricture at the anastomosis may be as high as 33%<sup>[4]</sup> making the resection of the affected liver sector an attractive alternative<sup>[4,18]</sup>. As we were not able to find and locate the injured duct during surgery in the first case, this was the only treatment option for our patient. Placement of a percutaneous, transhepatic catheter into the injured duct prior to surgery may help the surgeon identify the site of injury facilitating dissection of the hepatic hilum prior to reconstruction<sup>[4]</sup>. However, application of this technique on an undilated biliary system may be a challenge even

for an experienced hepatobiliary radiologist<sup>[16]</sup>. For this reason we did not decide to attempt this approach in our patient. While surgery remains the mainstay treatment in most centers, management of such injuries using the interventional radiology approach has also been reported. This includes the application of minimally invasive and percutaneous techniques of ablation of the affected duct by ethanol or acetic acid injection<sup>[19,20]</sup>.

In summary, proper diagnosis and management of right sectoral BDI remains difficult. It should always be suspected and looked for if a biliary leak following cholecystectomy persists despite its absence on endoscopic cholangiogram. In addition to a bilioenteric reconstruction with the Roux limb of jejunum, resection of the affected part of the liver is another treatment option.

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**L- Editor** A **E- Editor** Zhang DN



## Biliary ascariasis in a bile duct stones-removed female patient

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Author contributions: Wang J and Pan YL contributed equally to this work; Wang J performed ERCP; Pan YL collected, analyzed and interpreted data; Xie Y drafted the manuscript; Wu KC and Guo XG revised the manuscript; all authors critically reviewed and approved the manuscript.

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biliary colic, acute cholangitis, obstructive jaundice, choledocholithiasis and acute cholecystitis. Here, we describe a case with biliary ascariasis two days after endoscopic sphincterotomy for choledocholithiasis. A living ascaris was successfully removed by endoscopic retrograde cholangiopancreatography. This case indicated that biliary ascariasis is not an uncommon complication of endoscopic sphincterotomy in some regions where ascariasis is epidemic.

Wang J, Pan YL, Xie Y, Wu KC, Guo XG. Biliary ascariasis in a bile duct stones-removed female patient. *World J Gastroenterol* 2013; 19(36): 6122-6124 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6122.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6122>

### Abstract

Biliary ascariasis is a common problem in rural areas in China. The common presentations include biliary colic, acute cholangitis, obstructive jaundice, choledocholithiasis and acute cholecystitis. Here, we describe a case with biliary ascariasis two days after endoscopic sphincterotomy for choledocholithiasis. A living ascaris was successfully removed by endoscopic retrograde cholangiopancreatography. This case indicated that biliary ascariasis is not an uncommon complication of endoscopic sphincterotomy in some regions where ascariasis is epidemic.

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**Key words:** Biliary ascariasis; Endoscopic retrograde Cholangiopancreatography; Endoscopy; Choledocholithiasis

**Core tip:** Biliary ascariasis is a common problem in rural areas in China. The common presentations include

### INTRODUCTION

Biliary ascariasis is a common problem in rural areas in China, especially in the Northwest region. Common presentations include biliary colic, acute cholangitis, obstructive jaundice, choledocholithiasis and acute cholecystitis<sup>[1]</sup>. In this report we describe a case with biliary ascariasis after endoscopic sphincterotomy for choledocholithiasis.

### CASE REPORT

An 18-year-old Chinese woman presented with complaints of abdominal pain, fever and jaundice. Magnetic resonance cholangiopancreatography (MRCP) showed multiple common bile duct (CBD) stones (Figure 1). Routine endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic sphincterotomy were performed and several CBD stones were retrieved successfully. The recovery was uneventful until the onset of progressively intolerable abdominal pain two days later. Abdominal ultrasonography revealed a dilated common bile duct (10 mm) with an ill-defined linear echogenic shadow inside

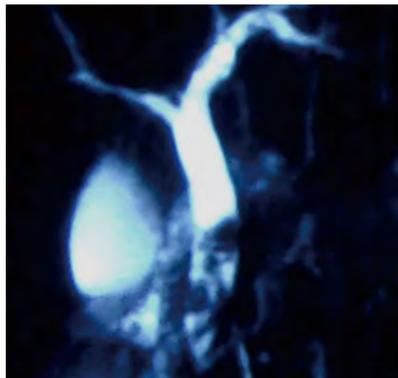


Figure 1 Magnetic resonance cholangiopancreatography showing multiple common bile duct stones.



Figure 2 Abdominal ultrasonography revealed a dilated common bile duct with an ill-defined linear echogenic shadow inside (arrow). CBD: Common bile duct.

(Figure 2). The gallbladder was normal. Biliary ascariasis was suspected and ERCP was repeated. The head of a worm was at the sphincterotomy-performed papilla (Figure 3). One living ascaris of about 25 cm in length was removed using a dormia basket (Figure 4).

## DISCUSSION

Endoscopic sphincterotomy is the treatment of choice for patients with choledocholithiasis. Its main complications include bleeding, pancreatitis, perforation and infection<sup>[2]</sup>. Biliary ascariasis, one of contributing factors of biliary stones, does not figure in the list. However, biliary ascariasis is a common complication of endoscopic sphincterotomy in some regions where ascariasis is epidemic. For example, Gupta *et al*<sup>[3]</sup> reported four cases of biliary ascariasis among 273 cases with post-endoscopic sphincterotomy for choledocholithiasis in Kashmir, India. Biliary ascariasis occurred only two days after sphincterotomy in the present study, which contrasts with the interval period of 2-24 wk in Gupta's report<sup>[3]</sup>.

Abdominal ultrasonography can facilitate the diagnosis of biliary ascariasis in most of cases. The characteristic sonographic feature of worms in the common bile duct is a long, linear, parallel echogenic strip, usually without acoustic shadowing<sup>[4]</sup>. ERCP, considered the gold standard for the diagnosis of biliary ascariasis, should be reserved for therapeutic rather than diagnostic use, because papillotomy may lead to reentry of the worm into the common bile duct<sup>[5]</sup>. In addition, sphincterotomy is not recommended unless a basket cannot be introduced inside the common bile duct to hold the ascaris<sup>[6]</sup>. Dead ascaris and stones in the same patients have been reported to be pulled out simultaneously through the CBD<sup>[6]</sup>. In this patient, CBD stones were removed successfully by the first ERCP and no signs of either living or dead biliary ascaris were found. The living ascaris found by the second ERCP was thought to have resulted from the entry of the worm into the common bile duct through the open papilla; therefore, anthelmintics were used to prevent reentry of any other ascaris residing in the small bowel. Six months of follow-up were uneventful.

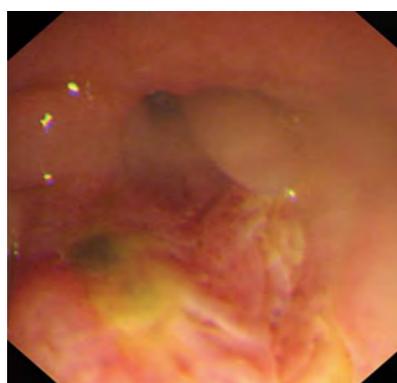


Figure 3 The head of a worm observed in the sphincterotomy-performed papilla.



Figure 4 A living ascaris of about 25 cm in length was removed using a dormia basket.

Previously, Zargar *et al*<sup>[7]</sup> described the endoscopic management of postoperative biliary ascariasis in 19 consecutive patients who underwent cholecystectomy and choledocholithotomy. ERCP was performed 4 to 16 d after biliary tract surgery and roundworms in the CBD were extracted from 10 patients, from the hepatic ducts of patients, or from both ducts in seven patients. Taken together, we suggest that endoscopic management is an effective and safe approach for extracting ascarids from

the biliary tree of postoperative patients.

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**L- Editor** Stewart GJ **E- Editor** Zhang DN



## Metachronous intracystic and intraductal papillary neoplasms of the biliary tree

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lesions within the lumen of the biliary tree including the gallbladder, papilla of Vater, and pancreatic duct have been proposed collectively as a preinvasive neoplastic lesion. The patient reported here was an interesting case of intraluminal papillary neoplasm involving the gallbladder and metachronously the extrahepatic bile duct. This letter to the editor encourages clinicians to detect more of such neoplastic lesions.

Sato H, Sato Y, Harada K, Sasaki M, Hirano K, Nakanuma Y. Metachronous intracystic and intraductal papillary neoplasms of the biliary tree. *World J Gastroenterol* 2013; 19(36): 6125-6126 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i36/6125.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i36.6125>

### Abstract

A 77-year-old woman complained of epigastralgia, and a tumor (5 cm in diameter) of the gallbladder neck was detected by image analysis. Following cholecystectomy, the tumor was pathologically diagnosed as intraductal papillary neoplasm (IPN), gastric type, with associated invasive carcinoma. About 10 mo later, intraluminal multiple masses (3 foci, up to 1.8 cm) were noted in the extrahepatic bile duct, and the resected specimen showed that all tumors had similar gross and microscopic features as seen in gallbladder IPN without invasion, and they were synchronous multiple lesions. This case showed a papillary tumor of the gallbladder of gastric phenotype, and confirmed that the gallbladder is a target of IPN in addition to the bile ducts.

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**Key words:** Intraductal papillary neoplasm; Intracystic papillary neoplasm; Gastric type; Metachronous occurrence; Synchronous occurrence

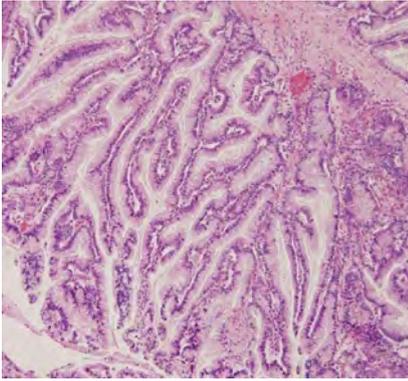
**Core tip:** Recently, the papillary or tumoral neoplastic

### TO THE EDITOR

The World Health Organization (WHO) Classification of Tumours of the Digestive System (2010) recognizes intraductal papillary neoplasms of the bile duct (IPNBs) as a precancerous entity of cholangiocarcinoma<sup>[1,2]</sup>. Before the recognition by WHO, this entity was called by various and many different names, such as biliary papilloma and papillomatosis, and papillary adenocarcinoma<sup>[2-4]</sup>. IPNBs are occasionally multicentric along the intrahepatic and extrahepatic bile duct, synchronously and metachronously.

Interestingly, IPNB shares many features with intraductal papillary mucinous neoplasms of the pancreas (IPMN-Ps). For example, four phenotypes of epithelium are recognized in IPNB as well as IPMN-P<sup>[1,5,6]</sup>: pancreatobiliary, intestinal, oncocytic, and gastric types. Intracystic papillary neoplasm (IPN) of the gallbladder was newly described in addition to papillary adenomas in the 2010 WHO classification.

We have recently experienced a case of IPN of the gallbladder followed by metachronous IPNBs of the extrahepatic bile duct. A 77-year old woman complained of epigastralgia, and a tumor of the gallbladder neck was



**Figure 1** Cauliflower tumor of the gallbladder shows papillary neoplasm composed of columnar epithelium with fine fibrovascular core, resembling gastric foveola (hematoxylin and eosin, x 100).



**Figure 2** Intraluminal masses of the extrahepatic bile ducts and dilatation of extrahepatic bile duct shown by endoscopic retrograde cholangiography.

detected by computed tomography (CT) and magnetic resonance imaging (MRI). By cholecystectomy, a papillary, cauliflower-like tumor (5 cm × 4.8 cm) growing intraluminally was found at the gallbladder neck, and the remaining gallbladder was distended. Most of the tumor showed a well-differentiated papillary neoplasm composed of columnar epithelium with supranuclear mucin, resembling gastric mucosa, particularly gastric foveola, covering fine vasculo-fibrous cores (Figure 1), and showed moderate- to high-grade intraepithelial neoplasia. There was focal invasion of moderately differentiated tubular adenocarcinoma in the gallbladder wall. There was no metastasis, and the gallbladder mucosa, except for the tumor, did not show dysplastic or metaplastic changes. The tumor was diagnosed as IPN of the gallbladder with associated invasive carcinoma.

About 10 mo later, intraluminal multiple masses of the extrahepatic bile duct and secondary dilatation of the intra/extrahepatic bile ducts were demonstrated by CT, MRI and endoscopic retrograde cholangiography (Figure 2). The affected extrahepatic bile duct was surgically resected. The resected bile duct was dilated and filled with three separate brown papillary masses (1.8 cm × 1.4 cm, 1.5 cm × 1.5 cm, and 1.2 cm × 1.1 cm). There was no mucin within the resected bile ducts. These extrahepatic tumors commonly showed similar gross and microscopic features as seen in gallbladder IPN, but no invasion. There were no significant pathological changes in the background of the extrahepatic bile ducts. There was no continuity between the three lesions and they were regarded as synchronous multiple lesions.

Immunohistochemically, MUC5AC and MUC6 were diffusely positive, whereas MUC2-positive cells were scattered in these IPNs, and MUC1 was not observed in any of the four IPNs of the gallbladder and bile ducts. These IPNs were regarded as gastric type.

Similar to IPNB, IPN of the gallbladder may present with a pancreatobiliary or intestinal phenotype<sup>[2,3]</sup>. Distin-

guishing IPN from papillary adenomas of the gallbladder may be controversial. The vast majority of IPNs of the gallbladder have a biliary phenotype, whereas papillary adenomas exhibit an intestinal or gastric phenotype. This case showed a papillary tumor of the gallbladder of gastric phenotype, and confirmed that the gallbladder is a target of IPN in addition to the bile ducts.

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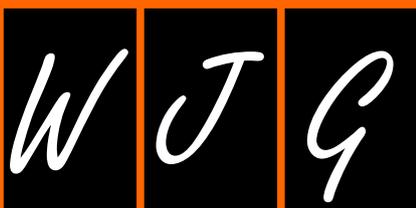
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## Strategy for improving survival and reducing recurrence of HCV-related hepatocellular carcinoma

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### Abstract

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related death in the world. With advances in imaging diagnostics, accompanied by better understanding of high-risk patients, HCC is now frequently detected at an early stage; however, the prognosis remains poor. The recurrence rate after treatment of HCC is higher than that associated with cancers of other organs. This may be because of the high incidence of intrahepatic distant recurrence and multicentric recurrence, especially with hepatitis C virus (HCV)-related hepatocellular carcinoma. The Barcelona Clinic Liver Cancer (BCLC) classification has recently emerged as the standard classification system for the clinical management of patients with HCC. According to the BCLC staging system, curative therapies (resection, transplantation, transcatheter arterial chemoembolization, percutaneous ethanol injection therapy, percutaneous microwave coagulation therapy and percutaneous radiofrequency ablation) can improve survival in HCC patients diagnosed at an early stage and offer a potential long-term cure. However, treatment strategies for recurrent disease are not mentioned in the BCLC classification. The strategy for recurrence may differ according to the recurrence pattern, *i.e.*, intrahepatic distant recurrence *vs* multicentric

recurrence. In this article, we review recurrent HCC and the therapeutic strategies for reducing recurrent HCC, especially HCV-related HCC.

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**Key words:** Hepatocellular carcinoma; Intrahepatic distant recurrence; Multicentric recurrence; Hepatitis C virus; Interferon; Arterial chemotherapy

**Core tip:** Recent advances in treatment modalities have improved the survival rate of patients with hepatocellular carcinoma (HCC). However, long-term outcomes of patients with HCC remain unsatisfactory because of the high incidence of distant intrahepatic recurrence, multicentric recurrence and low survival rates. In particular, hepatitis C virus-related hepatocellular carcinoma has a much higher recurrence rate than other cancers. In this article, we describe the prognosis of recurrent HCC and the therapeutic strategies for reducing recurrent HCC.

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer mortality in the world<sup>[1,2]</sup>. It is estimated that HCC is responsible for more than 600000 deaths annually worldwide<sup>[3]</sup>.

Recent advances in treatment modalities have improved the survival rate of patients with HCC<sup>[4,5]</sup>. However, long-term outcomes for patients with HCC remain unsatisfactory because of the high incidence of intrahepatic distant recurrence, multicentric recurrence and low

survival rates.

In many cases, surgical options for HCC are limited because of complicating hepatic cirrhosis; furthermore, HCC is associated with a 5-year recurrence rate of approximately 80% after radical treatment, which is much higher than that of other gastrointestinal carcinomas, resulting from its tendency to multicentric carcinogenesis secondary to chronic liver disease, or intrahepatic distant recurrence<sup>[6,7]</sup>.

Typically, recurrence rates in HCC follow a 2-peak distribution. Early recurrence usually occurs within 2 years after resection, and is most closely related to cancer metastasis, while late recurrence mainly results from *de novo* tumors as a consequence of the carcinogenic cirrhotic environment<sup>[7]</sup>. Therefore, a treatment strategy with a focus on recurrence is necessary.

In recent years, the Barcelona Clinic Liver Cancer (BCLC) classification has emerged as the standard classification system for the clinical management of patients with HCC<sup>[8]</sup>. However, in the recommendations regarding topical therapy for the treatment of early stage HCC, the BCLC guidelines do not mention a strategy for reducing recurrence. Togo *et al*<sup>[9]</sup> recommended a strategy based on the differentiation between recurrence types, *i.e.*, intrahepatic distant recurrences *vs* multicentric recurrence. Transcatheter arterial infusion (TAI) with platinum agents may be effective as adjuvant therapy for the prevention of residual liver recurrence after hepatectomy, probably by suppression of the development of intrahepatic micrometastasis, rather than multicentric carcinogenesis. Furthermore, antiviral treatment, including interferon (IFN), is recommended for preventing multicentric recurrence. We discuss a possible strategy for reducing the recurrence of hepatitis C virus (HCV)-related HCC in terms of our clinical data.

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## PREVENTIVE TREATMENT STRATEGY PRIOR TO DEVELOPMENT OF RECURRENT MULTIPLE HEPATOCELLULAR CARCINOMA TO MAINTAIN RESIDUAL HEPATIC FUNCTION

---

IFN therapy may be useful in the prevention of recurrence of HCC secondary to chronic viral hepatitis after radical treatment by inhibiting multicentric carcinogenesis, while chemotherapy based on TAI is recommended to inhibit intrahepatic metastasis.

It is widely recognized that HCV infection is a major cause of liver cirrhosis and HCC in Japan and other countries. According to the Liver Cancer Study Group of Japan, 67.7% of Japanese patients with HCC are HCV antibody-positive<sup>[10]</sup>.

We evaluated the treatment response and functional hepatic reserve in patients who received combination

therapy with PEG-IFN  $\alpha$ -2b and ribavirin (RBV) after radical treatment of HCV-related HCC<sup>[11]</sup>.

This study comprised 54 patients with primary HCV-related HCC (stage I / II) whose survival rate, meta-chronous recurrence rate and hepatic functional reserve were assessed. Among these patients, 29 received combination therapy with PEG-IFN  $\alpha$ -2b and RBV after treatment of HCC (secondary IFN treatment group), and the other 25 did not receive IFN, including PEG-IFN  $\alpha$ -2b and RBV (non-secondary treatment group). The 1- and 3-year cumulative survival rates were 100.0% and 90.2% in the secondary IFN treatment group, and 96.0% and 61.2% in the non-secondary treatment group, respectively, showing a significant difference between the groups. Multivariate analysis identified secondary IFN treatment as a significant factor related to prognosis. In the PEG-IFN  $\alpha$ -2b/RBV group, serum albumin levels decreased transiently but increased thereafter, indicating improvement in hepatic functional reserve. These results show that combination therapy with IFN, including PEG-IFN  $\alpha$ -2b and RBV, following treatment of HCC, contributes to improvement in hepatic functional reserve and increases treatment options in the case of recurrence.

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## TREATMENT STRATEGIES FOR INTRAHEPATIC METASTASIS

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For intrahepatic metastasis, on the other hand, it is essential to select treatment not only for overt lesions, but also for micrometastasis. Hence, combination therapy, including intra-arterial treatment, such as transcatheter arterial chemoembolization (TACE), is required for initial treatment or treatment of recurrence.

### ***Efficacy of platinum-containing drugs in transhepatic arterial infusion***

After treatment of HCC, the remaining liver is still in a state of precarcinogenesis. The protective effect of chemotherapy against recurrence in the remaining liver is demonstrated by its efficacy in patients with a high probability of intrahepatic metastasis, and thus, a high recurrence rate.

Although it is well-established that more effective chemotherapy, performed preoperatively in patients with intrahepatic micrometastasis or with the possibility of intraoperative tumor spread, may prevent tumor recurrence in the residual liver and further improve prognosis, few reports have described such cases. Systemic chemotherapy is generally not effective in most cases of HCC. Further, chemotherapy often impairs liver function in cases complicated by cirrhosis. At present, systemic chemotherapy with cytotoxic anticancer agents is only used infrequently in the treatment of HCC. Compared with systemic chemotherapy, hepatic arterial infusion (HAI) chemotherapy has the advantages of increasing the local concentration of chemotherapeutic agents to levels that are adequate to kill cancer cells without damaging healthy

liver tissue, and of reducing systemic side effects.

### **Treatment strategy and problems with TACE**

In Japan, TACE, which is recommended for inoperable patients in whom local puncture therapy is not indicated according to the guidelines for the management of liver cancer, plays a central role in the treatment of recurrent advanced multiple HCC<sup>[12,13]</sup>.

TACE is primarily performed as chemolipiodolization, using anticancer drugs mixed with lipiodol<sup>[14,15]</sup>. There have also been several retrospective reports of TACE with anthracyclines *vs* platinum compounds, suggesting the utility of platinum compounds<sup>[16,17]</sup>. However, no single effective drug has been identified, because no prospective comparative studies have been performed, and various anticancer drugs, including epirubicin, cisplatin, mitomycin C and doxorubicin, have been used with intercenter variance.

Kaibori *et al*<sup>[18]</sup> previously recommended that preoperative whole-liver chemolipiodolization reduces postoperative recurrence and prolongs survival in patients undergoing resection of hepatocellular carcinoma.

However, they subsequently performed a randomized controlled trial to evaluate the influence of preoperative TACE on survival after the resection of HCC, following which they concluded that preoperative selective TACE and whole-liver chemolipiodolization plus TACE do not reduce the incidence of postoperative recurrence or prolong survival in patients with resectable HCC<sup>[19]</sup>.

While intra-arterial chemotherapy is not highly appreciated in the West, HAI resulting in high local drug concentrations, is expected to improve the prognosis and prevent disease recurrence, because lesions are often localized to the liver even in advanced stages of HCC. However, various therapeutic regimens have been tried, without reaching a consensus regarding the administration method or dose level.

We previously reported that platinum agents, such as cisplatin, which are widely used for the treatment of a variety of malignancies, may be effective for HCC treatment.

In Japan, a fine powder formulation of cisplatin (cisplatin powder) (DDPH, IA call; Nippon Kayaku, Tokyo, Japan) was developed in 2004 and approved for the treatment of HCC *via* a transarterial approach, without lipiodol or embolic material. Cisplatin powder is readily soluble and more suitable for the preparation of high-concentration (about three times) aqueous solutions (1.4 mg/mL) than conventional cisplatin formulations (0.5 mg/mL). Therefore, a single session of TAI therapy with cisplatin powder has the benefit of increasing drug concentration locally in the HCC, and is expected to have a high therapeutic efficacy.

We evaluated the effectiveness of additional chemotherapy with the platinum-containing drugs carboplatin (CBDCA) and DDPH in preventing intrahepatic distant tumor recurrence<sup>[20]</sup>.

Seventy-eight patients with a diagnosis of primary stage I / II HCC who underwent TACE and RFA after

whole liver arterial infusion of CBDCA (25 patients) or DDPH (53 patients) for local control and recurrence prevention were followed up on a long-term basis. The clinical background factors, intrahepatic distant tumor recurrence rate, and intrahepatic distant tumor recurrence factors were compared between the CBDCA and DDPH groups. While no significant differences in background clinical characteristics were observed between the two groups, the intrahepatic distant tumor recurrence rate was significantly lower in the DDPH group<sup>[20]</sup>. In multivariate analysis using Cox's proportional hazard model, whole liver arterial infusion of DDPH was identified as an independent factor for the prevention of recurrence, *i.e.*, whole liver arterial infusion of DDPH significantly prevented intrahepatic distant tumor recurrence. Significant prevention of recurrence by a single infusion of DDPH compared with CBDCA suggests the utility of DDPH-based treatment strategies in patients with intrahepatic metastasis.

However, no evidence of its contribution to patient survival has been found, and TAI is not mentioned in any Western guidelines<sup>[21]</sup>. Nevertheless, it has been shown that some patients with TACE-refractory HCC were responsive to repeated TAI, with survival being prolonged in these responsive patients. According to the 18<sup>th</sup> nationwide follow-up survey of primary liver cancer, 85.8% of 1862 Japanese HCC patients treated with chemotherapy underwent TAI<sup>[10]</sup>. TAI is relatively often selected as the final choice for advanced HCC, including recurrent HCC; therefore, it is important to improve the response rate to TAI.

Thus, treatment strategies with DDPH-based TAI need to be established by conducting prospective randomized control trials<sup>[22,23]</sup>.

## **CONCLUSION**

Currently, the efficacy of tertiary prevention of HCC with any agent, including chemotherapy, HCV therapy, or IFN, has yet to be proven, and safe and effective chemotherapy for HCC-recurrence has yet to be established.

In this article, we reviewed our strategy for improving survival and reducing the recurrence of HCV-related HCC.

When deciding on treatment strategies for recurrence of HCV-related HCC, it is very important to select appropriate treatment according to the degree of disease progression, and to determine the patient's functional hepatic reserve. The type of recurrence and previous treatments also should be taken into consideration. It is also imperative to establish preventive strategies against precarcinogenesis associated with multicentric carcinogenesis and residual intrahepatic metastasis as early as possible, thereby improving prognosis.

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## Transjugular intrahepatic portosystemic shunt for the management of acute variceal hemorrhage

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### Abstract

Acute variceal hemorrhage, a life-threatening condition that requires a multidisciplinary approach for effective therapy, is defined as visible bleeding from an esophageal or gastric varix at the time of endoscopy, the presence of large esophageal varices with recent stigmata of bleeding, or fresh blood visible in the stomach with no other source of bleeding identified. Transfusion of blood products, pharmacological treatments and early endoscopic therapy are often effective; however, if primary hemostasis cannot be obtained or if uncontrollable early rebleeding occurs, transjugular intrahepatic portosystemic shunt (TIPS) is recommended as rescue treatment. The TIPS represents a major advance in

the treatment of complications of portal hypertension. Acute variceal hemorrhage that is poorly controlled with endoscopic therapy is generally well controlled with TIPS, which has a 90% to 100% success rate. However, TIPS is associated with a mortality of 30% to 50% in such a setting. Emergency TIPS should be considered early in patients with refractory variceal bleeding once medical treatment and endoscopic sclerotherapy failure, before the clinical condition worsens. Furthermore, admission to specialized centers is mandatory in such a setting and regional protocols are essential to be organized effectively. This review article discusses initial management and then focuses on the specific role of TIPS as a primary therapy to control acute variceal hemorrhage, particularly as a rescue therapy following failure of endoscopic approaches.

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**Key words:** Cirrhosis; Portal hypertension; Transjugular intrahepatic portosystemic shunt; Variceal hemorrhage

**Core tip:** The transjugular intrahepatic portosystemic shunts (TIPS) is a highly effective treatment for bleeding esophageal and gastric varices with control of the bleeding in over 90% of the patients. Many papers have been published in the last decade that led to technical improvements and definition of the best indications for this promising treatment of complications of portal hypertension. The purpose of this article is to describe the different treatment options for patients with refractory esophageal and gastric varices bleeding and the role of TIPS as a rescue therapy. Technical aspects of this procedure and the current indications are also discussed.

Loffroy R, Estivalet L, Cherblanc V, Favelier S, Pottecher P, Hamza S, Minello A, Hillon P, Thouant P, Lefevre PH, Krausé D,

Cercueil JP. Transjugular intrahepatic portosystemic shunt for the management of acute variceal hemorrhage. *World J Gastroenterol* 2013; 19(37): 6131-6143 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6131.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6131>

## INTRODUCTION

Acute variceal hemorrhage is a common clinical emergency and most often is caused by cirrhosis-related portal hypertension<sup>[1]</sup>. Less common causes include splenic vein thrombosis, hepatic veno-occlusive disease, and primary biliary cirrhosis<sup>[1]</sup>. It is defined as visible bleeding from an esophageal or gastric varix at the time of endoscopy, the presence of large esophageal varices with recent stigmata of bleeding or fresh blood visible in the stomach with no other source of bleeding identified<sup>[1]</sup>. The frequency of gastroesophageal varices in cirrhosis varies from 30% to 70% with bleeding occurring in approximately one-third of patients<sup>[2]</sup>. Twenty percent of cirrhotics with acute variceal hemorrhage die within 6 wk<sup>[3]</sup>. The rebleeding rates range from 30% to 40% at 6 wk and the mortality from rebleeding reaches 30%<sup>[4]</sup>. Gastroesophageal varices account for approximately 80% of all cases of variceal hemorrhage<sup>[2,5]</sup>. The precipitating cause for hemorrhage, presumably an acute rise in portal pressure and subsequent variceal rupture, remains uncertain. However, several factors have been implicated including raised intra-abdominal pressure, bacterial infection, continued excess alcohol consumption and postprandial increase in splanchnic blood flow<sup>[4,5]</sup>. Predictive factors for variceal hemorrhage include a hepatic venous pressure gradient (HVPG) of > 20 mmHg<sup>[6,7]</sup>, the presence of large varices with red signs<sup>[8]</sup> and underlying severe liver disease (Child-Pugh grade C)<sup>[2]</sup>.

Optimal management of variceal hemorrhage requires a multidisciplinary approach involving a team of gastroenterologists, hepatologists, critical care physicians, surgeons, and interventional radiologists. The principal components of therapy include airway maintenance, hemodynamic stabilization, control of the variceal hemorrhage, and alteration of the hemodynamic effects of portal hypertension. Treatment options for the management of acute variceal hemorrhage include endoscopic therapy, use of vasoactive drugs, balloon tamponade and esophageal transaction. These various methods, either alone or in combination, are effective in controlling acute variceal hemorrhage in 80% to 90% of patients<sup>[3]</sup>. Patients who do not respond to these measures are referred for rescue therapies, which include transjugular intrahepatic portosystemic shunt (TIPS) and surgical portosystemic shunts with or without splenectomy. Because of the higher mortality of surgery in the acute setting, TIPS is the favored rescue procedure for uncontrolled variceal hemorrhage<sup>[6]</sup>.

The purpose of this review is to describe the different therapeutic options available to control acute variceal hemorrhage and then to focus on the potential role of

TIPS as a primary therapy to control acute variceal hemorrhage, particularly as a rescue therapy following failure of endoscopic approaches.

## INDICATIONS-GASTROINTESTINAL BLEEDING

TIPS has been used to treat many complications related to portal hypertension. The relative efficacy of TIPS has been tested with randomized controlled trials (variceal bleeding, refractory ascites), whereas other indications have been evaluated in uncontrolled case series.

The causes of gastrointestinal hemorrhage in a patient with portal hypertension may be variceal rupture, portal hypertension gastropathy, postsclerotherapy ulcers, peptic ulcer disease, hemorrhagic gastritis, and Mallory-Weiss tear. TIPS is generally accepted as a second-line therapy after failure of endoscopic and medical therapy of bleeding from gastroesophageal varices<sup>[9]</sup>.

### Primary prophylaxis of variceal bleeding

Bleeding from esophageal varices is a common and severe complication of portal hypertension. Prevention of the initial bleeding can be achieved in a number of cases by endoscopic variceal ligation or  $\beta$ -blocker treatment. However, TIPS has never been tested in this situation as the use of surgical portacaval shunts has demonstrated that this approach is associated with higher morbidity and mortality rates<sup>[10]</sup>.

Bleeding from gastric varices is often severe and difficult to control, particularly when fundal varices are involved. The first-line treatment is endoscopic sclerotherapy with cyanoacrylate<sup>[11]</sup>. TIPS has been used in a number of uncontrolled trials in patients in whom endoscopic therapy failed<sup>[12,13]</sup>. A recent controlled trial has shown that TIPS is more efficient than cyanoacrylate in prevention of rebleeding (secondary prophylaxis) from large gastric varices<sup>[14]</sup>. This finding, although interesting, must be confirmed by after a long-term follow-up. Importantly, due to the large size of fundal varices, the risk of rupture is still present even at a low portacaval gradient (< 12 mmHg) after TIPS<sup>[15]</sup>. This is probably best explained by the relationship between the variceal tension (and therefore the risk of rupture) and the variceal size. For this reason, it is recommended to embolize gastric varices at the time of TIPS placement<sup>[10,16]</sup>.

### Acute variceal bleeding

When initial bleeding occurs, it is usually controlled with less invasive endoscopic treatment and/or pharmacological therapy. In the rare instance when bleeding remains uncontrollable, TIPS has been used as a rescue treatment with good results. However, prognosis relies on the general condition of the patient, the value of the liver function reserve, and the associated comorbidities<sup>[17-20]</sup>. However, a recent randomized controlled trial evaluated the use of emergent TIPS as compared to standard medical therapy in patients with severe portal hypertension and a

**Table 1** Transjugular intrahepatic portosystemic shunt *vs* endoscopic therapy in the prevention of rebleeding: Results from meta-analyses *n* (%)

Study finding	Reference, value	
	Burroughs <i>et al</i> <sup>[34]</sup>	Zheng <i>et al</i> <sup>[35]</sup>
Patients	948	883
TIPS	472	440
Endoscopic therapies	476	443
Randomized controlled trials	13	12
Recurrent bleeding		
TIPS	88 (18.6)	86 (19.0)
Endoscopic therapy	210 (44.1)	194 (43.8)
OR (95%CI) for TIPS	0.30 (0.21-0.44)	0.32 (0.24-0.43)
Post-treatment encephalopathy		
TIPS	134 (28.4)	148 (33.6)
Endoscopic therapy	83 (17.4)	86 (19.4)
OR (95%CI) for TIPS	2.08 (1.49-2.94)	2.21 (1.61-3.03)
All-cause mortality		
TIPS	130 (27.5)	111 (25.2)
Endoscopic therapy	118 (24.8)	98 (22.1)
OR (95%CI) for TIPS	1.14 (0.85-1.54)	1.17 (0.85-1.61)

TIPS: Transjugular intrahepatic portosystemic shunt.

Child-Pugh score of 7-13<sup>[21]</sup>. Treatment failure was more frequent in the medical group (50% *vs* 12%) and the survival rate was better in the TIPS group (11% *vs* 38%)<sup>[21]</sup>. This approach could justify the use of TIPS early after bleeding in patients with moderate or severe liver failure and severe portal hypertension. Current evidence supports the use of TIPS not as a primary form of treatment, but rather as a rescue treatment for patients with bleeding esophageal varices who failed pharmacological and endoscopic treatments.

### Secondary prophylaxis of variceal bleeding

The strongest evidence in favor of performing a TIPS procedure exists for the secondary prevention of variceal bleeding. Twelve randomized controlled trials have been published on this topic, describing results for 948 patients, 472 of whom received a TIPS<sup>[22-33]</sup>. Recent meta-analyses found a more than threefold decrease in the risk of recurrent bleeding after insertion of a TIPS compared with various forms of endoscopic therapy (Table 1)<sup>[34,35]</sup>. Rates of rebleeding after insertion of TIPS ranged from 9.0% to 40.6%. Conversely, continued endoscopic therapy resulted in a 20.5% to 60.6% rate of rebleeding. All-cause mortality rates were similar between the TIPS and endoscopic therapy groups. However, there was a more than twofold increase in the rate of development of hepatic encephalopathy after a TIPS procedure<sup>[22-33]</sup>.

### Ectopic varices

Varices may develop anywhere along the digestive tract in patients with portal hypertension (duodenum, jejunum, colon, rectum) and may bleed. Local treatments are either impossible or associated with a high rate of rebleeding. The best approach is the TIPS procedure, which can be combined with embolization of the varices<sup>[36,37]</sup>.

**Table 2** Contraindications to placement of a transjugular intrahepatic portosystemic shunt

Absolute	Relative
Congestive heart failure	Portal vein thrombosis
Severe pulmonary hypertension	Hepatocellular carcinoma
Severe systemic sepsis	Severe coagulopathy
Unrelieved biliary obstruction	Hepatic encephalopathy
Severe tricuspid regurgitation	Obstruction of all hepatic veins

### Portal hypertensive gastropathy

These gastric lesions rarely induce problematic bleeding. Nonetheless, anecdotal case reports have suggested that TIPS may control bleeding in these patients<sup>[38]</sup>.

### Gastric antral vascular ectasia

Chronic bleeding from gastric antral vascular ectasia may be difficult to manage. However, TIPS does not help to control this type of hemorrhage, probably because these vascular lesions are related to liver disease and not to portal hypertension<sup>[38,39]</sup>.

### Other indications

Despite limited evidence, TIPS has found wider clinical use than just secondary prevention of variceal bleeding, treatment of refractory acute variceal bleeding and management of refractory ascites. These clinical indications include Budd-Chiari syndrome<sup>[40,41]</sup>, hepatic veno-occlusive<sup>[42]</sup>, hepatic hydrothorax<sup>[43-46]</sup>, hepatorenal syndrome<sup>[47,48]</sup>, and hepatopulmonary syndrome<sup>[49]</sup>.

## CONTRAINDICATIONS

Absolute contraindications to TIPS include right heart failure and pulmonary arterial hypertension. The TIPS survival benefit in patients with severe liver failure (Child-Pugh class C cirrhosis, model for end-stage liver disease score > 22, serum bilirubin > 3 mg/dL) also remains unclear. Relative contraindications include hepatic encephalopathy (which may worsen following TIPS creation), polycystic liver disease (technically challenging with a high incidence of hemorrhagic complications), active sepsis (poor outcomes), and chronic organized portal vein thrombosis (technically challenging for successful TIPS creation). Acute portal vein thrombus is not a contraindication for TIPS, but it necessitates extensive stenting to prevent shunt occlusion<sup>[50]</sup>. The contraindications are summarized in Table 2.

## PRE-TIPS TREATMENT OPTIONS FOR ACUTE VARICEAL BLEEDING

### Initial management

As with all acutely unwell patients, the basic resuscitation pathway (airway, breathing, circulation) should be instigated. Initially, the airway and breathing should be

assessed. Endotracheal intubation should be considered early, especially in patients who are deemed at high risk for aspiration, that is, those demonstrating signs of encephalopathy or ongoing severe uncontrolled hemorrhage. The adequacy of filling of the circulation should then be assessed and two large bore intravenous canula inserted before placement of a central line. Plasma expanders and packed red blood cells should be used to replace volume loss and any underlying coagulopathy corrected with platelets and fresh-frozen plasma. Despite portal pressure correlating directly with plasma volume, all cirrhotic patients with variceal hemorrhage should be maintained at a normal central venous pressure, while avoiding under filling the circulation in order to “keep the portal pressure low”<sup>[51]</sup>. Ideally, these patients should be admitted to an intensive care or high dependency unit where cardiac monitoring and high intensity nursing are readily available. All patients with cirrhosis and gastrointestinal bleeding are at an increased risk of bacterial infection and thus prophylactic antibiotics should be administered<sup>[52,53]</sup>. Several meta-analyses have demonstrated a reduction in bacterial infections and improved survival attributed to the use of short-term prophylactic antibiotics<sup>[54]</sup>. No consensus exists as to which antibiotic should be given but intravenous quinolones are generally recommended for 5-7 d followed by oral quinolones<sup>[55-57]</sup>.

### Endoscopic therapy

Sclerotherapy and variceal band ligation are the two endoscopic interventions currently used. Endoscopic sclerotherapy involves a sclerosant such as ethanolamine injected directly into the bleeding varix. Variceal band ligation is associated with fewer side effects than sclerotherapy. Banding devices that allow multiple bands to be applied without repeated reintroduction of the endoscope should be used. Variceal band ligation is the preferred endoscopic therapy for the secondary prevention of esophageal variceal hemorrhage and most centers now also use band ligation to control acute bleeding<sup>[58]</sup>.

### Pharmacological treatment

Various pharmacological agents, including vasopressin, somatostatin, octreotide and terlipressin, are of benefit in acute variceal bleeding<sup>[59-61]</sup>. These drugs cause splanchnic vasoconstriction and thus reduce portal flow. They are particularly useful when an out-of-hours endoscopy service is unavailable. Temporary cessation of bleeding and reduction in treatment failure has been reported with early administration of these drugs<sup>[62]</sup>. An ongoing debate does continue about the efficacy of these agents, particularly vasopressin analogues, as they are not without significant side effects such as increased risk of mesenteric ischemia and myocardial infarction. These agents should therefore be used with caution in patients with known atheromatous disease. Vasopressin is no longer used alone and rarely with nitrates, with terlipressin being the current agent of choice. Because a significant proportion

of patients suspected of variceal hemorrhage will actually be bleeding from nonvariceal sources, widespread use of vasoactive drugs before endoscopy should be discouraged, as is diagnostic endoscopy attempted by someone who is unable to perform band ligation or sclerotherapy. Combination therapy of these vasoactive agents and endoscopic therapy is becoming common but a meta-analysis of several studies, although demonstrating initial improvement in hemostasis, did not reveal a reduction in mortality with combination therapy<sup>[63]</sup>.

### Balloon tamponade

Balloon tamponade is invaluable in cases of uncontrolled hemorrhage when an endoscopy service is unavailable or when control cannot be achieved endoscopically. Balloon tamponade, however, is not without complications that include gross esophageal ulceration and esophageal perforation. To minimize complication rates, this procedure should be performed only by experienced staff and in the majority of cases, lone inflation of the gastric balloon should be sufficient. In the rare cases that the esophageal balloon requires inflation, inflation pressure should be closely monitored and regular deflation should also be performed. Nursing protocols should be produced and should include regular checks of the gastric balloon position and regular aspiration from both the gastric and esophageal ports. Medical staff should be alerted if blood aspiration volumes are increasing at either port. Panés *et al.*<sup>[64]</sup> examined the use of esophageal tamponade in 151 cases and reported that although balloon tamponade achieved hemostasis, 50% of patients experienced rebleeding on removal of the Stenstaken-Blackmore tube. It is essential therefore that balloon tamponade is considered only as a holding measure until a definitive procedure can be performed.

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## TIPS PROCEDURE

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### Timing of salvage therapy

Although the above studies illustrate the efficacy and applicability of TIPS in the setting of uncontrolled variceal bleeding, there remains a debate about the best time to perform the procedure. Although a convenient definition of uncontrolled variceal bleeding can be taken as failure of two endoscopic treatments, this does not necessarily indicate criteria for TIPS insertion. Patients with a Child-Pugh A score and whose bleeding does not appear life threatening may be managed by balloon tamponade followed by further sessions of endoscopic band ligation and generally do not require TIPS. Conversely, patients with advanced liver disease who have had a single massive bleed and unsuccessful endoscopic treatment on one occasion and require balloon tamponade, may be better treated by TIPS early rather than undergoing a second endoscopic therapy session. Monescillo *et al.*<sup>[65]</sup> showed that early insertion of TIPS might confer extra benefit. The basis of this is probably due to reducing the duration

or risk of hypotension that is likely to be detrimental for patients with decompensated liver disease.

### **Pre-procedural imaging**

Any prior imaging studies (ultrasound, computed tomography, magnetic resonance imaging), should be reviewed to confirm portal vein patency and to assess the presence of gastroesophageal varices and other porto-systemic shunts that may compete with the TIPS. The location of the portal vein bifurcation should be determined based on prior imaging, as an extrahepatic portal vein bifurcation occurs in 25% of patients and accessing an extrahepatic portal vein during TIPS carries a high mortality<sup>[10,66]</sup>. Imaging may also demonstrate the presence of splenic vein thrombosis, for which TIPS is not the treatment of choice, ascites, and general hepatic morphology. If there is large-volume ascites, pre-procedural paracentesis should be performed. If no imaging is available, Doppler ultrasound assessment of the portal vein is recommended before initiating the TIPS procedure<sup>[66]</sup>. The procedure is performed under general anesthesia and thus an emergency consultation with anesthesia is initiated as soon as TIPS is considered.

### **Equipment specifications**

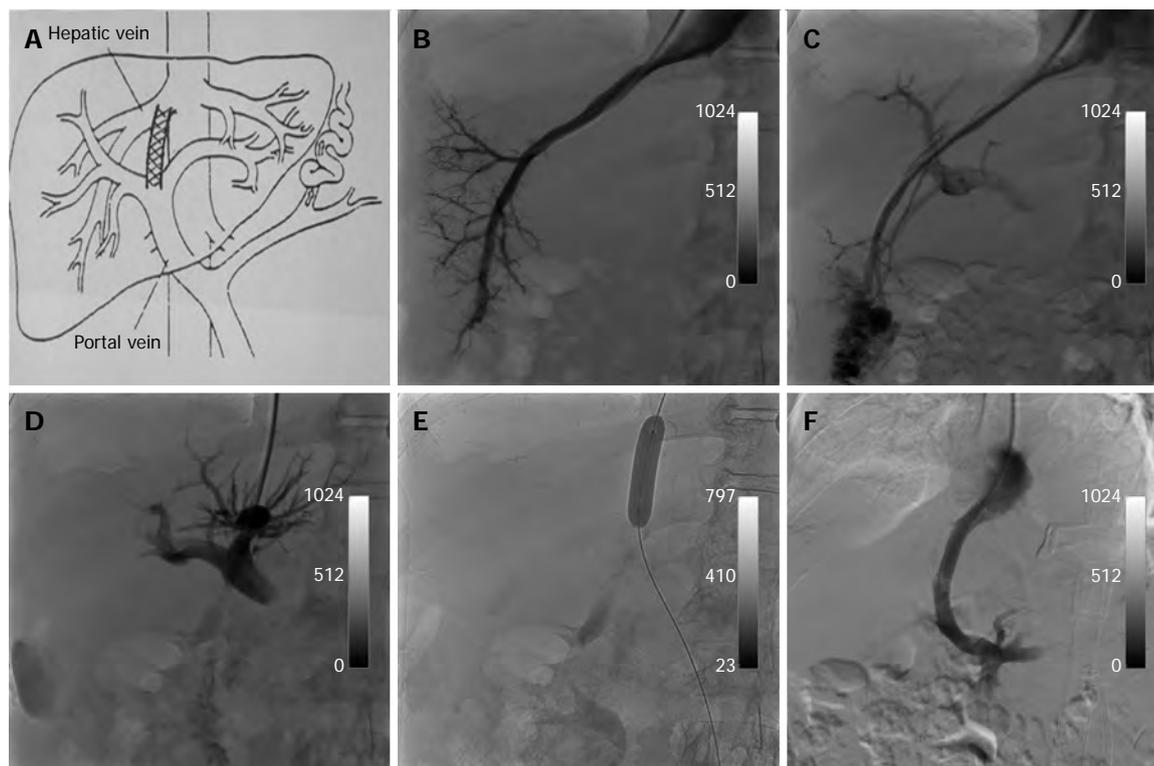
The procedure room should have the necessary equipment for continuous hemodynamic monitoring as well as for anesthesia, with access to oxygen, anesthetic gases, and suction. The angiographic equipment should allow for high-resolution fluoroscopy, digital subtraction angiography (DSA), and operator-definable protocols for performing CO<sub>2</sub> DSA, low-frame-rate fluoroscopy, and road map imaging. A trained radiologic technologist who is familiar with the necessary catheters, guidewires, balloons, stents, and imaging equipment should be present. Anesthesia or nursing personnel are essential for patient monitoring and assistance with hemodynamic measurements. The physician operator should be an interventional radiologist who is trained in performing TIPS procedures, as these require a high level of technical expertise and knowledge of the equipment, materials, anatomy, physiology, pathology, appropriate technique, and potential complications. The operator must be able to cope with the difficulties that are often associated with emergency TIPS<sup>[6,9,50,66]</sup>.

### **Shunt technique**

**Sets:** Three types of TIPS sets are commercially available. Two sets, made by Cook Medical (Bloomington, IN, United States), include the “RingTIPS set” and the “Rosch-Uchida TIPS set”. The RingTIPS set has a 16-G curved Colapinto needle, while the Rosch-Uchida set has a 16-G curved blunt cannula through which a 5-Fr catheter with an inner needle is advanced to access the portal vein. After using the needle to advance the catheter, the needle is removed and the catheter is slowly withdrawn while maintaining suction in the catheter. There is also a cope version of the ring set, which uses a 20- to 21-G-

long needle. Another set is made by AngioDynamics (Queensbury, NY, United States) and has a hollow 21-G needle that is passed through a hollow, curved cannula.

**Steps:** After entry into the internal jugular vein, a catheter is introduced and guided through the superior vena cava, right atrium, and inferior vena cava into a hepatic vein. The use of the proximal portion of the hepatic vein has two purposes. The first is to utilize, for shunt creation, the largest diameter of the hepatic vein to potentially prevent or delay any outflow shunt stenosis. The second is to be sure that one begins cephalad to the desired portal vein entry site. A needle inserted through the catheter is then used to puncture the liver from a central portion of the hepatic vein and enter the main portal branch, usually the right portal vein. In the right hepatic vein, the cannula is rotated approximately 90° anteriorly and then advanced and maintained with continual caudal pressure, so that it is wedged against the wall of the hepatic vein. When in the middle hepatic vein, the cannula is rotated posteriorly in the same way. Carbon dioxide wedged hepatic venography is used to identify the portal vein<sup>[67]</sup>. Iodinated contrast medium can also be used. The puncture can be also navigated with ultrasonography. Depending on the anatomy, it might be possible to use a tract from the right hepatic vein to the left portal branch, and vice versa. The needle tract is then dilated by a balloon catheter, establishing a connection between the portal and systemic circulation directly inside the liver parenchyma. The parenchymal tract is kept open by insertion of an expandable metallic stent. A dedicated TIPS stent graft was designed to extend the covered portion to the orifice of the hepatic vein at the inferior vena cava<sup>[41]</sup>. The only uncovered part of the stent graft, which is 2 cm long, is the section that protrudes into the portal vein. This both anchors the device and allows blood to flow through the interstices of the uncovered portion to the peripheral (parenchymal) portal vein branches. The alternative to the dedicated stent graft has been a self-expandable stent used for bridging portal and hepatic veins in a similar way. The bare stents are used for patients at high risk of hepatic encephalopathy or for recanalization of the portal vein. The shunt diameter is finalized by balloon dilatation of the deployed stent graft or stent. Depending on the diameter of the expandable stent or stent graft used for TIPS creation, various amounts of portal blood are diverted into the systemic circulation, resulting in the decompression of portal hypertension. The size of the balloon catheter is usually 8 mm. Depending on the pressure gradient measured between the portal vein and right atrium after stent or stent graft placement, a larger angioplasty balloon catheter is an option to achieve adequate stepwise decompression. For liver transplant candidates, precise positioning of both ends of the stent or stent graft is critical<sup>[6,50]</sup>. The needle may exit the liver and lacerate the liver capsule or enter the hepatic artery. Embolization of the parenchymal tract is the first-line



**Figure 1** Conventional transjugular intrahepatic portosystemic shunt creation technique. A: Schematic diagram shows transjugular intrahepatic portosystemic shunt (TIPS) connecting the right hepatic vein to the right portal vein. The shunt extends from main portal vein to confluence of right hepatic vein and inferior vena cava; B: Right hepatic venogram shows course of hepatic vein; C: Transhepatic portogram using iodinated contrast material shows course of portal veins; D: Injection of contrast medium through Colapinto needle confirms needle position within portal vein before passage of guidewire; E: Dilatation of a tract through the hepatic parenchyma that is interposed between the hepatic and portal veins; F: Portal venogram obtained after TIPS insertion shows flow through the FLUENCY polytetrafluoroethylene-covered stent. Peripheral portal vein branches are no longer opacified because of reversal of flow.

treatment to prevent hemoperitoneum. The TIPS tract must be intraparenchymal, or dilatation of the extrahepatic portion of the portal vein results in fast exsanguination, a complication that occurs in approximately 1% of procedures. Entry into the right or left portal vein branch should be at least 1 to 2 cm from the portal vein bifurcation. The direct injection into the dilated tract should be done as soon as possible to reveal potential extravasation. If it is positive, the balloon is again inflated and the stent graft placed to tamponade the extrahepatic leak. According to the patient's blood pressure, fluid volume resuscitation is immediately initiated and the anesthesiologist is called<sup>[6,9,50,66]</sup>. The final step of the TIPS procedure is placement of pigtail catheter over the portal vein guide wire for follow-up portography and blood pressure measurement within the main portal vein. Once the value is stabilized and recorded, the tip of the sheath or pigtail catheter is moved to the hepatic vein or the suprahepatic inferior vena cava, and the blood pressure is again recorded. Thus, at the completion of the TIPS procedure, at least four pressure values will have been obtained: those in the portal vein and hepatic vein (or inferior vena cava) before and after shunt placement. The different steps are summarized in Figure 1.

### Embolization of varices

Embolization of the esophageal varices at the time of

the TIPS is easily accomplished but its routine application has been also controversial. While embolization after TIPS occurs in 24% to 48% of patients<sup>[68,69]</sup>, it is not clear whether the combination of TIPS and variceal embolization is more effective than TIPS alone. Some authors recommend transjugular embolization of the varices to increase the effect of the shunt with respect to acute hemostasis<sup>[68,70]</sup>, and other authors do not perform embolization<sup>[71]</sup>. In our clinical practice, we perform embolization of varices only if we observe persistent contrast flow into the varices in the control portography after TIPS. Variceal embolization is also indicated for patients with recurrent esophageal bleeding despite a patent shunt<sup>[68]</sup>. Embolization of esophageal varices is most commonly performed with the use of metallic coils, but the use of liquid agents such as opacified enbucrilate and ethanol have also been described<sup>[17]</sup>. The use of absolute ethanol is not recommended due to the possible adverse effects including cardiovascular collapse due to the possible venous channels between the portal system and the pericardium, mainly from the pericardiophrenic vein.

### Post-procedural follow-up

Recurrence or worsening of the portal hypertension symptoms should prompt an ultrasound with Doppler to exclude TIPS stenosis. Shunt velocities between 50 and 250 cm/s are associated with high (> 90%) sensitiv-

**Table 3** Acute and chronic complications after transjugular intrahepatic portosystemic shunt placement

Acute complications	Acute complications	Chronic complications
Minor or moderate Stent displacement	Life-threatening Hemobilia	Portal vein thrombosis
Neck hematoma	Hemoperitoneum	Congestive heart failure
Arrhythmia	Cardiac failure	Progressive liver failure
Shunt thrombosis	Liver ischemia	Chronic recurrent encephalopathy
Hepatic vein obstruction	Sepsis	Stent dysfunction

ity and specificity for shunt dysfunction<sup>[72]</sup>. In addition, most hepatologists order routine TIPS surveillance tests at regular intervals using ultrasound with Doppler in asymptomatic patients. Patients with a suspected TIPS dysfunction should undergo TIPS venography. If the original TIPS was created using a bare-metal stent, placement of a covered stent is likely to improve long-term shunt patency<sup>[73]</sup>. Other commonly used measures include balloon angioplasty within the stents and the placement of additional stents in patients to extend cranial or caudal length of the stent. Hepatic encephalopathy refractory to medical management or progressive hepatic dysfunction after TIPS placement might require endovascular shunt reduction. A commonly used technique involves shunt catheterization by two parallel guidewires followed by simultaneous deployment of two stents within the shunt. One of the stents is a covered endograft through which blood flow will be conducted, whereas the second device is a balloon-expandable bare-metal stent, the diameter of which determines the ultimate shunt diameter. Usually, the bare-metal stent is placed along the cephalic aspect of the covered stent. This allows continued access to the balloon expandable stent if further reduction is necessary<sup>[74,75]</sup>.

## OUTCOMES

### Complications

The TIPS procedure may lead to a number of adverse events (Table 3). Technical complications sustained at the time of TIPS placement can include transcapsular puncture, which may occur in as many as 33% of cases<sup>[10]</sup>. The capsular perforation leads to significant intraperitoneal hemorrhage 1% to 2% of the time<sup>[10]</sup>. Clinically significant hemobilia is also rarely observed after the procedure. The stent can be placed too far into the inferior vena cava or even into the right atrium at the cranial end or far into the main portal vein at the caudal end of the shunt in up to 20% of patients<sup>[10]</sup>. On occasion, stents may migrate because of catheter and balloon manipulation<sup>[76]</sup>. Diversion of portal venous flow through the shunt diminishes the metabolic filtering effect of the hepatic parenchyma, leading to new or worsened encephalopathy in 30% to 46% of patients<sup>[34,35]</sup>. Chronic recurrent disabling hepatic encephalopathy can occur in 5% to 10% of patients and may lead to a complete loss of the patient's autonomy<sup>[10,66]</sup>. Several pre-TIPS parameters have been tested to

**Table 4** Risk factors for post-transjugular intrahepatic portosystemic shunt encephalopathy

Risk factors
Age
Sex
Cause
Child-Pugh score
Hepatic encephalopathy history TIPS
Porto-hepatic gradient
Stent diameter
Indication
Creatinine

TIPS: Transjugular intrahepatic portosystemic shunt.

predict post-TIPS hepatic encephalopathy (Table 4)<sup>[10,34,35]</sup>. Deterioration of hepatic function in approximately 10% of patients<sup>[35]</sup>, and hepatorenal syndrome is occasionally observed<sup>[77]</sup>. TIPS stenosis and occlusion was the method of choice before wide acceptance of PTFE-covered stents (Viatorr; W.L. Gore and Associates, Flagstaff, AZ, United States). The most common site of shunt stenosis is at the hepatic venous end. The culprit of midstent stenosis is thought to be intimal hyperplasia within the bare-metal stent due to contact between traversed biliary radicles and stent lumen<sup>[76]</sup>. The incidence of stenosis due to hyperplasia within the stent ranged from 18% to 78%<sup>[76]</sup> for bare-metal stents, which led to recurrence of portal hypertension complications and required frequent invasive procedures for reconstitution of flow. The introduction of PTFE-covered stent grafts led to dramatic improvement in long-term TIPS patency. A randomized controlled trial published in 2007 established a PTFE-covered stent as the preferred device for TIPS<sup>[78]</sup>. In that study, 80 patients were randomized to receive either a covered ( $n = 39$ ) or a bare ( $n = 41$ ) metal stent and were followed for two years after TIPS placement. Compared with patients treated with a bare-metal stent, patients with a PTFE-covered stent had a significantly lower rate of TIPS dysfunction (15% *vs* 44%), a higher rate of primary patency (76% *vs* 36%), a lower rate of clinical relapse (10% *vs* 29%), and were less likely to develop encephalopathy (33% *vs* 49%)<sup>[78]</sup>. On the basis of these data, a PTFE-covered stent became the standard of care device for de novo TIPS. Patients who have a bare metal stent TIPS should undergo shunt revision with a PTFE-covered stent in the event of shunt dysfunction<sup>[76]</sup>.

### Mortality

Acute variceal hemorrhage that is poorly controlled with endoscopic therapy is generally well controlled with TIPS, which has a success rate of 90% to 100%. However, TIPS also has a mortality rate of 27% to 50%<sup>[19,66,79,80]</sup>. Increased mortality is related to a Child-Pugh C clinical status, hemodynamic instability at the time of the TIPS procedure, and the presence of other comorbidities. In general, early TIPS intervention allows for better control of hemorrhage with decreased mortality. Patients with a

high HVP ( $> 20$  mmHg) and acute variceal bleeding have a better survival with TIPS than with endoscopic therapy<sup>[65]</sup>. Most of the deaths of patients after emergency TIPS are related to hepatic failure, multiorgan failure, and sepsis, often accompanied by variceal and nonvariceal bleeding, while only a minority are related to recurrent variceal bleeding<sup>[13,69,81]</sup>. Death occurring within 30 d of the procedure is most commonly caused by multiorgan failure, and death more than 30 d following the procedure is most commonly related to liver failure<sup>[81]</sup>. Many studies reporting on emergency TIPS for the rescue treatment of acute esophageal varices bleeding have shown low survival rates and significantly higher mortality rates than patients undergoing elective TIPS<sup>[6,12,17,19,21,65]</sup>. In one study, 42 of 123 (34.1%) of patients died within 30 d of TIPS for acute bleeding, while only 16.5% died following elective TIPS creation<sup>[82]</sup>. As an independent predictor of mortality, patients bleeding at the time of TIPS creation were 2.9 times more likely to die than those associated with elective TIPS placement. Similar findings have been reported by Helton *et al.*<sup>[83]</sup> who reported a 56% in-hospital mortality rate for patients who were actively bleeding or hemodynamically unstable at the time of the TIPS *vs* 5.5% following nonemergency procedures. The reported mortality associated with TIPS varies widely because the inclusion criteria, timing of the TIPS, and the severity of liver disease. Many reports combine the results of patients actively bleeding during TIPS with those of the patients who were stable during the procedure. Several reports describing different prognostic factors associated with mortality after TIPS have been published<sup>[68,69,82]</sup>. Prognostic factors are not intended to predict outcome or management on individual basis or to deny a patient a potentially lifesaving intervention, but are useful as guidelines to develop appropriate expectations and to weigh different therapeutic options. Final decisions are based on the individual patient needs and overall clinical condition<sup>[65,84]</sup>. Many of these prognostic factors correlate with the mortality of patients undergoing elective TIPS. In patients with acute variceal bleeding, however, these predictors may fail because the hepatic reserve and renal function are difficult to evaluate in the acute setting<sup>[65]</sup>. Events such as bleeding, infection, and high-dose diuretic therapy may affect the renal and liver function in a transient way. No single prognostic criterion is available to accurately select patients with a very high risk of death<sup>[85]</sup>. However, several selection criteria have been described due to an increased amount of experience within the field with relation to TIPS<sup>[86]</sup>.

### **Effect on liver and spleen stiffness**

Variceal bleeding still remains the major cause of death in patients with cirrhosis, with increasing numbers of inpatient cases with advanced liver disease and portal hypertension. For those patients, TIPS has become the rescue treatment of choice, preferred over liver transplantation. Therefore, it is crucial to ensure that the inserted TIPS effectively decreases portal vein pressure to prevent

variceal bleeding. Non-invasively assessing the pressure of the portal vein as a function of the TIPS has been a challenge. Color Doppler sonography can measure flow velocities in the TIPS, but it cannot reflect the pressure of the portal vein and its pitfalls and inaccuracies lead to a lack in necessary sensitivity<sup>[87]</sup>. More recently, a novel ultrasound-based acoustic radiation force impulse (ARFI) elastography has been developed that can provide information on the local mechanical property of a tissue<sup>[88]</sup>. An acoustic push pulse transmitted by the transducer toward the tissue produces an elastic shear wave that propagates through the tissue. The propagation of the shear wave is followed by detection pulses that are used to measure the velocity of the shear wave propagation, which is directly related to tissue elasticity. In other words, the speed of shear wave is dependent on the elasticity of the tissue<sup>[88]</sup>.

Gao *et al.*<sup>[89]</sup> prospectively assessed the stiffness of the liver and spleen with ARFI imaging pre- and post-TIPS placement. The investigators measured stiffness of the liver and spleen with mean shear wave velocity (MSV, m/s) on ARFI imaging for 10 healthy volunteers and 10 patients who underwent TIPS placement for treatment of portal hypertension. The portal vein pressure was measured during TIPS placement. A significant difference in portal vein pressure was found for the pre- ( $27.67 \pm 5.86$  mmHg) and post- ( $18.00 \pm 6.93$  mmHg) TIPS insertion. Significant differences were also found in MSV of the liver and spleen between healthy subjects and patients with portal hypertension. There was no significant difference found in MSV of the liver pre- and post-TIPS placement. However, a statistically significant difference in MSV of the spleen pre- and post-TIPS placement was demonstrated. In addition, the authors reported a significant difference in spleen index between healthy subjects and patients with portal hypertension, as well as between pre- and post-TIPS placement. The MSV of the spleen measured with ARFI correlated well with portal vein pressure. Hence, the authors concluded that spleen stiffness determined by means of MSV on ARFI imaging could be used as a quantitative marker for monitoring the portal vein pressure as the function of the TIPS.

In this study, as well, the authors had prospectively shown a close correlation between the stiffness of the spleen and portal vein pressure. Based on these data, one can clearly note that the stiffness of the spleen measured with MSV changes as the portal vein pressure changes following TIPS placement. This is the first quantitative demonstration of the effectiveness of TIPS on the stiffness of the spleen measured with MSV value on ARFI imaging. One parameter that was not significantly affected by TIPS placement was the MSV value of the liver. The most plausible explanation for this finding is that TIPS can have a direct impact on the pressure of the portal vein but have no effect on the stiffness of a fibrotic liver. The tissue mechanical property of a cirrhotic liver is very hard due to the severe fibrosis developed in the liver parenchyma, which has poor elasticity. In addition, MSV of the spleen has potential to serve as an indicating

marker with which to assess portal vein pressure. Finally, it may be used as a non-invasive predictor in screening for recurrent portal hypertension when TIPS malfunction develops.

### Economic benefit

Early insertion of TIPS in high-risk patients with acute variceal hemorrhage reduces rebleeding and mortality. However, the economic benefit of utilizing this approach remains unclear. Harman *et al*<sup>[90]</sup> retrospectively carried out a cost-effectiveness analysis of patients who may benefit from early TIPS insertion. The costs were calculated in a 12-mo follow-up from index bleeding admission and compared to a theoretical 12-mo follow-up cost related to early TIPS insertion. Over one year, 78 patients were admitted with variceal hemorrhage; 27 patients (35%) were eligible for early TIPS insertion. The actual cost for the 12-mo follow-up was £138473.50. The authors estimated early TIPS insertion would save £534.70 per patient per year ( $P < 0.0001$ ). According to sensitivity analysis, early TIPS was the dominant treatment modality up to a theoretical rebleeding rate of 6%, and the economic threshold of £15000 per bleeding episode saved was achieved at a 12% yearly rebleeding rate, suggesting it would be financially viable to adopt early TIPS as an intervention up to a 12% yearly rebleeding rate. This study indicates strict patient selection is vital to reduce the rebleeding rate when utilizing early TIPS insertion. There is an important balance between selecting patients at high risk of rebleeding, who are likely to benefit from early TIPS insertion to prevent rebleeding, but also to exclude patients with the most severe hepatic dysfunction where early TIPS insertion is unlikely to alter the natural history of their disease. Strict patient selection reduces rebleeding-related admissions, thus reducing follow-up costs; this is a key concept for centers to focus on before introducing early TIPS as routine practice. Finally, Harman *et al*<sup>[90]</sup> found 35% of their bleeding cohort were eligible for early TIPS insertion, further establishing early TIPS insertion as a cost-effective intervention. This has important implications for the future provision and organization of interventional radiology services. Future prospective studies evaluating early TIPS insertion are warranted, and including similar economic modeling will help to confirm the financial viability of introducing early TIPS insertion into routine clinical practice.

### CONCLUSION

The TIPS procedure is now a well-established treatment for complications of portal hypertension. Technical advances and well-designed clinical studies provide a scientific basis to define the best indications. Patients with acute variceal bleeding with a Child-Pugh score  $> 12$ , APACHE score II  $> 18$  points, hemodynamically unstable, receiving vasopressors and coagulopathy, and/or bilirubin  $> 6$  mg/dL have a high risk of early death after TIPS. In specific, in some individual clinical situations

it may be wise to withhold the TIPS because the mortality rate will be very high regardless of the therapy given. Every effort should be taken to stabilize the patient before TIPS, including the use of tamponade tubes and aggressive correction of coagulopathy. Once medical treatment and sclerotherapy fail, emergency TIPS should be considered early before the clinical condition worsens. Patients at high risk for early mortality after TIPS should be considered for expedited liver transplantation if available. Cost analysis must be performed in the future taking into account recent developments including technical improvements, better patient selection, and better post-TIPS management.

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## Molecular targeted therapy for hepatocellular carcinoma: Current and future

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### Abstract

Hepatocellular carcinoma (HCC) is one of the most frequent tumors worldwide. The majority of HCC cases occur in patients with chronic liver disease. Despite regular surveillance to detect small HCC in these patients, HCC is often diagnosed at an advanced stage. Because HCC is highly resistant to conventional systemic therapies, the prognosis for advanced HCC patients remains poor. The introduction of sorafenib as the standard systemic therapy has unveiled a new direction for future research regarding HCC treatment. However, given the limited efficacy of the drug, a need exists to look beyond sorafenib. Many molecular targeted agents that inhibit different pathways involved in hepatocarcinogenesis are under various phases of clinical development, and novel targets are being assessed in HCC. This review aims to summarize the efforts to target molecular components of the signaling pathways that are responsible for the development and progression of HCC and to discuss perspectives on the future direction of research.

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**Key words:** Hepatocellular carcinoma; Targeted therapy; Molecular agents; Sorafenib

**Core tip:** Sorafenib is the first drug to prolong survival of patients with advanced hepatocellular carcinoma (HCC). This advance has shifted the paradigm of systemic treatment for HCC toward molecular targeted therapy. This review aims to summarize the efforts to target molecular components of the signaling pathways that are responsible for the development and progression of HCC and to discuss perspectives on the future direction of research.

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is a common solid cancer and the third most frequent cause of cancer-related mortality worldwide. The 5-year relative survival rate for patients with HCC is only 7%, and very few patients with symptomatic disease survive for > 1 year<sup>[1]</sup>. One of the primary reasons for the poor prognosis of patients with HCC is the lack of effective treatment options, especially for those with advanced disease. Although surgery and percutaneous ablation can achieve long-term control in some patients with early HCC, fewer than 40% of patients are diagnosed at early stages; hence, only a minority of HCC patients are eligible for potentially curative therapies, such as resection, transplantation, or percutaneous ablation<sup>[2]</sup>. Furthermore, systemic therapies (such as stan-

dard chemotherapeutic agents) do not provide significant efficacy in HCC based on randomized trials<sup>[3]</sup>.

In recent years, improved knowledge of the oncogenic processes and signaling pathways that regulate tumor cell proliferation, differentiation, angiogenesis, invasion and metastasis has led to the identification of several potential therapeutic targets, which has driven the development of molecularly targeted therapies. An ideal cancer target meets the following criteria: (1) the target is relatively specific for cancer cells (not expressed or expressed at very low levels in normal cells but overexpressed in cancer cells). Meanwhile, overexpression of the target is associated with malignant biological phenotypes and/or poor prognosis; (2) inhibition of the target is efficacious (the target plays an essential role in cancer initiation and progression, and inhibition of expression or activity of the target induces growth suppression and/or apoptosis in cancer cells); and (3) the target is “drugable” as an enzyme (*e.g.*, a kinase) or a cell surface molecule (*e.g.*, a membrane-bound receptor) that can be easily screened for small-molecule inhibitors or targeted by a specific antibody<sup>[4]</sup>.

The aim of this article is to review the efforts to target molecular components of the signaling pathways that are responsible for the development and progression of HCC and to summarize the evidence for the clinical activity of these agents in patients with HCC.

## HCC DEVELOPMENT AND SIGNALING PATHWAYS

Hepatocarcinogenesis is a multistep process initiated by external stimuli that lead to genetic changes in hepatocytes or stem cells, resulting in proliferation, apoptosis, dysplasia and neoplasia. The majority of HCC cases are related to chronic viral infections. However, the mechanisms by which hepatitis B virus (HBV) or hepatitis C virus (HCV) induce malignant transformation seem to differ. HBV DNA integrates into the host genome, inducing chromosome instability and insertional mutations that may activate various oncogenes, such as cyclin A<sup>[5-7]</sup>. Viral proteins, in particular X protein (HBx), act as transactivators to upregulate several oncogenes (such as *c-myc* and *c-jun*) and transcriptional factors [(such as nuclear factor- $\kappa$ B)]<sup>[8-10]</sup>. Additionally, HBx activates promoters of genes encoding IL-8, tumor necrosis factor (TNF), transforming growth factor (TGF)- $\beta$  and epidermal growth factor receptor (EGFR)<sup>[11]</sup>. HBx can also stimulate several signal transduction pathways, including the JAK/STAT, RAS/RAF/MAPK, and Wnt/ $\beta$ -catenin pathways<sup>[12-14]</sup>.

The contributions of HCV to hepatocarcinogenesis are mediated by viral proteins, including core, NS3 and NS5A proteins. HCV core protein can promote apoptosis or cell proliferation through interaction with p53 or upregulation of Wnt-1 at the transcriptional level<sup>[15,16]</sup>. NS4A and NS4B proteins mediate translational inhibition and degradation of various cellular proteins<sup>[17]</sup>. Cirrhosis

is present in approximately 80%-90% of HCC patients and constitutes the largest single risk factor. In cirrhotic liver, changes in fat metabolism associated with the activation of adipocyte-like pathways are thought to be involved in neoplastic transformation<sup>[18]</sup>.

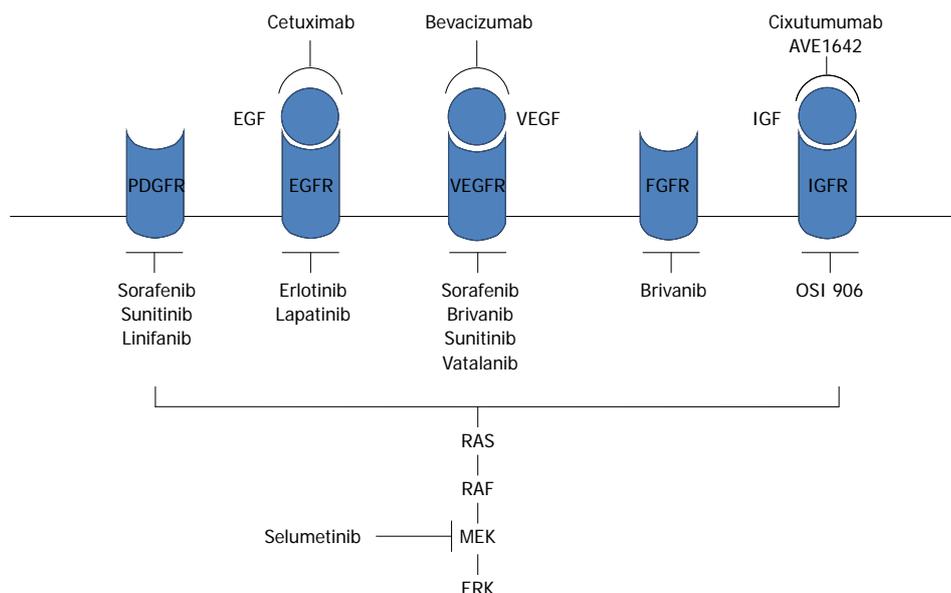
## MAPK PATHWAY (RAS/RAF/MEK/ERK)

The Raf/MAPK/extracellular-signal-regulated kinase (ERK) pathway is an important pro-survival signaling pathway that is primarily involved in cell growth and survival and regulates cell differentiation. This pathway transduces extracellular signals from membrane-bound tyrosine kinase receptors, such as EGFR, insulin-like growth factor receptor (IGFR), vascular endothelial growth factor receptor (VEGFR), *c-Met* and platelet-derived growth factor receptor (PDGFR), to the nucleus. Growth factor binding results in receptor phosphorylation, which activates an adapter molecule complex known as GRB2/SHC/SOS. This sequence in turn activates the RAF/mitogen/extracellular protein kinase (MEK)/ERK pathway, which triggers a cascade of specific phosphorylation events<sup>[19]</sup>. Within this pathway, the small GTPase RAS and the serine/threonine kinase Raf are the key signal regulators<sup>[20]</sup>. Intermediate signaling is regulated by MEK1 and MEK2, which are responsible for phosphorylating and activating the final downstream signaling molecules extracellular-regulated protein kinases (ERK)1 and ERK2<sup>[21]</sup>. ERK1/2 regulates cellular activity by acting on more than 100 substrates in the cytoplasm and nucleus. RAS also regulates the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, the phospholipase C/protein kinase C pathway and the *ral* guanine nucleotide dissociation stimulator pathway<sup>[22,23]</sup>.

Up-regulated activation of the Raf/MAPK/ERK signaling pathway has been well documented in HCC and correlates with advanced stage<sup>[24,25]</sup>. Mechanisms for the increased activity of the Raf/MAPK/ERK signaling pathway in HCC include down-regulation of Raf kinase inhibitor protein (a suppressor of the Raf/MAPK/ERK pathway) and induction by hepatitis viral proteins (such as the hepatitis B X protein and the hepatitis C core protein)<sup>[26-28]</sup>.

Targeting Raf kinase is one of the most promising targeted approaches for the treatment of HCC. Sorafenib has strong inhibitory activity against Raf-1 (C-Raf) kinase and B-Raf (wild-type B-Raf and mutant V600E B-Raf) and has been shown to inhibit other serine/threonine kinases, the pro-angiogenic receptor tyrosine kinases VEGFR, PDGFR and FGFR1, and tyrosine kinases such as *c-kit*, Flt-3 and RET, which are involved in tumor progression and overall prognosis (Figure 1)<sup>[29]</sup>.

Selumetinib (AZD6244) is an oral non-ATP-competitive small-molecule inhibitor of the mitogen-activated protein kinase MEK1/2. A recent study has shown that selumetinib plus rapamycin exerts antitumor and antiangiogenic effects in preclinical models of human HCC<sup>[30]</sup>. In a phase I / II study of selumetinib in combination with



**Figure 1** Ras/Raf/MEK/ERK signaling pathways and molecular targeted agents which is currently available or in development for hepatocellular carcinoma. EGF: Epidermal growth factor; EGFR: EGF receptor; ERK: Extracellular signal-regulated kinase; FGFR: Fibroblast growth factor receptor; IGF1R: Insulin-like growth factor receptor; PDGFR: Platelet-derived growth factor receptor; VEGF: Vascular endothelial growth factor; VEGFR: VEGF receptor; MEK: Mitogen/extracellular protein kinase.

sorafenib in advanced HCC, the objective responses were 3 partial response (PR), 6 stable disease (SD) and 2 progressive disease (PD) among 11 patients, and the common toxicities were diarrhea, rash, fatigue, and hypertension<sup>[31]</sup>.

Another phase I / II study has evaluated the combination of the MEK inhibitor RDEA119 and sorafenib in patients with advanced cancer (NCT00785226).

### PI3K/AKT/MTOR PATHWAY

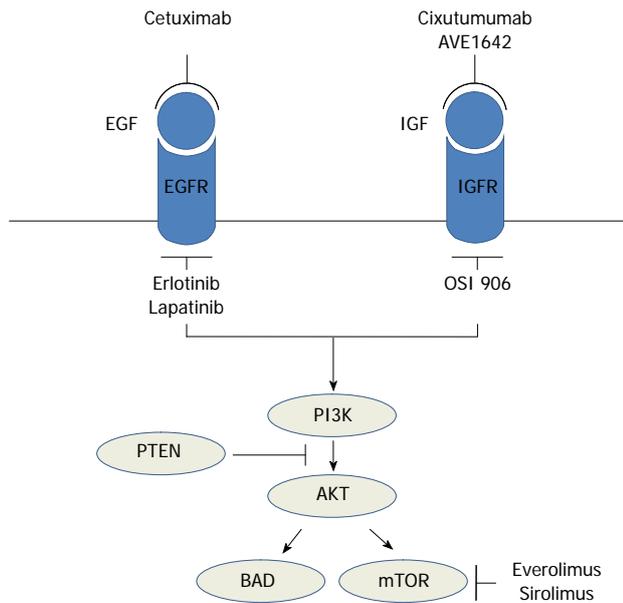
The PI3K/Akt/mTOR pathway also plays an important role in cell growth, survival regulation, metabolism and anti-apoptosis<sup>[32]</sup>. The binding of growth factors (such as IGF and EGF) to their receptors activates PI3K<sup>[19]</sup>. PI3K subsequently produces the lipid second messenger PIP3b (phospho-inositol triphosphate), which in turn activates the serine/threonine kinase AKT. Activated AKT also phosphorylates several cytoplasmic proteins, most notably mTOR and BCL-2-associated death promoter<sup>[19]</sup>. The activation of mTOR increases cellular proliferation, and inactivation of BAD decreases apoptosis and increases cell survival<sup>[21]</sup>. In normal tissue, this pathway is negatively regulated by the tumor suppressor phosphatase on chromosome 10 [phosphatase and tensin homolog (PTEN)], which targets the lipid products of PI3K for dephosphorylation<sup>[21]</sup>.

Expression of both IGF and the IGF receptor is up-regulated in HCC and cirrhotic liver, resulting in stimulation of the PI3K/AKT/mTOR signaling pathway in addition to activation of the RAF/MEK/ERK and WNT/ $\beta$ -catenin pathways<sup>[33,34]</sup>. Anomalies in PTEN function may lead to overactivation of the PI3K/AKT/mTOR pathway in HCC. PTEN expression is reduced in nearly half of all HCC tumors, resulting in constitutive

activation of the PI3K/AKT/mTOR pathway<sup>[35]</sup>. Decreased PTEN expression has been shown to correlate with increased tumor grade, advanced disease stage and reduced overall survival (OS) in patients with HCC<sup>[35]</sup>. In a mutation analysis of HCC samples, activation of the IGF pathway, upregulation of EGF, dysregulation of PTEN, and aberrant mTOR signaling were present in half of the samples; inhibition of mTOR activity with a rapamycin analog (everolimus) was effective in improving survival and suppressing recurrence<sup>[36]</sup>. These results suggest that mTOR pathway activation plays a crucial role in the pathogenesis of HCC (Figure 2).

The PI3K inhibitor RG7321 and the Akt inhibitor perifosine target the PI3K/Akt/mTOR pathway and are in early stages of clinical development. The mTOR inhibitors everolimus (RAD001), sirolimus (Rapamune) and temsirolimus (CCI-779) have been studied for efficacy and safety in patients with advanced HCC. Everolimus has produced a median progression-free survival (PFS) of 3.8 mo and OS of 8.4 mo in phase I / II testing in patients with advanced HCC<sup>[37]</sup>. A phase III study to compare everolimus and placebo and a phase I / randomized phase II study (sorafenib + everolimus *vs* sorafenib alone) to test the efficacy and tolerance of sorafenib in combination with everolimus are underway (NCT01035229). In a phase II study of sirolimus in patients with advanced HCC, sirolimus exhibited some antitumor activity in patients with advanced HCC<sup>[38]</sup>. However, larger studies are required to determine the value of this agent.

Temsirolimus, an mTOR inhibitor, has been approved for the treatment of advanced renal cell carcinoma. The efficacy and potential utility of this agent in HCC is currently under investigation (NCT01079767).



**Figure 2** PI3K/Akt/mTOR pathway and the molecular agents targeting this pathway. BAD: BCL-2-associated death promoter; EGF: Epidermal growth factor; EGFR: EGF receptor; IGFR: Insulin-like growth factor (IGF) receptor; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; PI3K: Phosphatidylinositol-3-kinase.

## VEGF/VEGFR, PDGFR, AND FGFR

Normal angiogenesis is maintained by a balance between proangiogenic and anti-angiogenic factors<sup>[39]</sup>. The angiogenic balance is disturbed in HCC. Angiogenesis is important for HCC growth and metastasis and occurs as a result of complex alterations that involve promoting factors [such as VEGF, angiopoietin and fibroblast growth factor (FGF), inhibitory factors, including thrombospondin (TSP) and angiostatin], and the surrounding tissue. A number of angiogenic growth factors, including VEGF-A, angiopoietin-2 and PDGF, have been shown to be upregulated in HCC tumors at the gene expression level and plasma protein level in patients with HCC compared with cirrhotic patients<sup>[40]</sup>. The principal angiogenic factors involved are VEGFs, PDGFs, TGF- $\alpha$  and - $\beta$ , basic FGF, EGF, hepatocyte growth factor (HGF), angiopoietins and interleukin-4 and -8<sup>[39,41]</sup>. These growth factors and cytokines induce angiogenic signaling through a variety of mechanisms, including activation of the RAF/MEK/ERK, PI3K/AKT/mTOR and JAK/signal transducer and activator pathways.

Increased VEGF expression has been reported in cirrhotic and dysplastic liver tissue, suggesting a possible role for VEGF-mediated angiogenesis in hepatocarcinogenesis<sup>[42]</sup>. VEGF clearly plays an important regulatory role in HCC; high levels of VEGF expression have been linked with HCC tumor grade, poor outcome after resection, disease recurrence, poor disease-free survival (DFS) and OS, vascular invasion and portal vein emboli<sup>[43-46]</sup>. Expression of FGF-2 is also elevated in patients with HCC, and FGF-2 expression in HCC correlates with

tumor microvessel density and postoperative recurrence rate<sup>[47-49]</sup>. Tumor angiogenic expression correlates with microvascular density in patients with HCC, and high serum angiogenic levels are associated with decreased survival at 5 years<sup>[50]</sup>.

The VEGF pathway can be targeted through two approaches: anti-VEGF monoclonal antibodies or inhibitors of the receptor tyrosine kinase associated with VEGFR. The anti-VEGF monoclonal antibody bevacizumab was the first angiogenesis inhibitor to be approved as an antineoplastic agent. Bevacizumab has shown encouraging early evidence of efficacy in patients with advanced HCC<sup>[51,52]</sup>. The combination of bevacizumab with either cytotoxic agents (gemcitabine, oxaliplatin, and capecitabine) or erlotinib has also shown encouraging results in four phase II trials in patients with advanced HCC<sup>[53-56]</sup>.

Sorafenib is an orally available multikinase inhibitor that was originally designed to target VEGFR-1, -2, -3, PDGFR and c-kit. In a phase II study of patients with advanced inoperable HCC, sorafenib induced a PR in 2% of the patients. The median time to progression (TTP) was 4.2 mo and median OS was 9.2 mo<sup>[57]</sup>. In the phase III SHARP (Sorafenib HCC Assessment Randomized Protocol) trial, sorafenib (400 mg twice daily) significantly prolonged OS compared with placebo in patients with advanced HCC (10.7 mo in the sorafenib group *vs* 7.9 mo in the placebo group)<sup>[58]</sup>. The median time to radiological progression was significantly longer in the sorafenib group (5.5 mo *vs* 2.8 mo). In another randomized phase III study performed in the Asia-Pacific region, the OS was 6.5 mo in the sorafenib group compared with 4.2 mo in the placebo group (the hazard ratio in the sorafenib group was 0.68,  $P = 0.014$ )<sup>[59]</sup>. Sorafenib is the only targeted therapy to have been approved for clinical use in several countries, including the United States and in Europe. Although sorafenib improved OS in patients with HCC, the associated toxicities may significantly affect patients' quality of life. High rates of dermatologic side effects have commonly been reported with sorafenib, the most clinically significant of which is hand-foot skin reaction<sup>[60]</sup>. Despite initial responses to sorafenib, most HCC patients experience a loss of efficacy. No effective second-line treatment options currently exist for patients who are resistant or refractory to and/or intolerant of sorafenib.

Beyond sorafenib, sunitinib is the most studied multikinase inhibitor targeting VEGFR-1 and VEGFR-2. Sunitinib also displays inhibitory activities against other receptor tyrosine kinases, including PDGFR-a/b, c-KIT, FLT3, and RET kinases. Sunitinib is currently indicated for the treatment of renal cell carcinoma and gastrointestinal stromal tumors<sup>[61-63]</sup>. Two phase II studies of sunitinib in patients with advanced HCC have been performed. In the first study, the PR rate was 2.9%, and 50% of the patients achieved stable disease<sup>[64]</sup>. In a second phase II study, one (2.7%) patient experienced a confirmed PR and 13 (35%) of 37 patients achieved stable

disease<sup>[65]</sup>. A phase III study comparing sunitinib with sorafenib (NCT00699374) was discontinued due to a greater incidence of adverse events in the sunitinib group and because sunitinib failed to demonstrate superiority or non-inferiority to sorafenib in extending the survival of patients with advanced HCC.

Brivanib is a dual inhibitor of VEGFR and fibroblast growth factor receptor signaling pathways. Because FGF signaling may contribute to acquired “resistance” or compensatory signaling during anti-VEGFR therapy, the simultaneous inhibition of these 2 pathways by brivanib may both delay initial progression in response to antiangiogenic therapy (as first-line treatment) and successfully treat tumors that have already progressed during the course of anti-VEGFR therapy (as second-line treatment)<sup>[66,67]</sup>. Brivanib has demonstrated a disease control rate of 51% and an OS of 10 mo as first-line monotherapy in a phase II trial of predominantly Asian patients with HCC<sup>[68]</sup>. In another phase II trial of brivanib in patients with HCC who had been treated with sorafenib, brivanib caused a tumor response rate of 4.3% and disease control rate of 45.7%<sup>[69]</sup>.

Large randomized phase III Brivanib Study in Patients at Risk (BRISK) HCC program trials have been conducted to evaluate the role of brivanib in advanced HCC (BRISK-FL, BRISK-PS and BRISK-APS). The BRISK-PS trial evaluated brivanib *vs* placebo in patients who had failed or were intolerant to sorafenib therapy (NCT00825955). This study did not meet its primary end point of improving OS, but treatment with brivanib showed improvements in the response rate<sup>[70]</sup>. The BRISK-FL trial (NCT00858871) directly compared the clinical outcomes of brivanib *vs* sorafenib in patients with advanced HCC who received no prior systemic therapy. The median OS was 9.5 mo in the brivanib arm compared with 9.9 mo in the sorafenib arm, which was not a statistically significant difference. No significant survival differences were observed between subgroups based on geographic regions, cause of HCC or disease severity. The study did not meet its primary OS objective based upon a non-inferiority statistical design<sup>[71]</sup>.

Vatalanib (PTK787) is a potent tyrosine kinase inhibitor that binds directly to the ATP-binding sites of VEGF receptors. Vatalanib inhibits both Flt-1 and Flk-1/KDR and other class III receptor tyrosine kinases, such as PDGFR- $\beta$ , Flt-4, c-kit, and c-fms<sup>[72]</sup>. In a phase I / II study of vatalanib combined with doxorubicin in patients with advanced HCC, the overall response rate was 26.0%, with all of the responding patients achieving PR. Another 20% of the patients achieved SD for at least 12 wk<sup>[73]</sup>.

Linifanib (ABT-869) is a novel receptor tyrosine kinase inhibitor with potent activity against members of the VEGFR and PDGFR families<sup>[74]</sup>. In a phase II study of linifanib in advanced HCC, the estimated objective response rate was 9.1%, the median time to disease progression was 3.7 mo, and the median OS was 9.7 mo<sup>[75]</sup>. An open-label, randomized phase III study of the efficacy

and tolerability of linifanib *vs* sorafenib in advanced HCC (NCT01009593) was conducted. The OS of linifanib given as monotherapy once daily was similar to sorafenib given twice daily per standard of care<sup>[76]</sup>.

TSU-68 is an oral tyrosine kinase inhibitor of FGFRs, VEGFRs and PDGFR and has demonstrated some clinical efficacy in a phase I / II trial of heavily pretreated patients with advanced HCC. Treatment of patients with unresectable or metastatic HCC with TSU-68 was associated with disease stabilization or improvement in 51% of the patients<sup>[77]</sup>. A randomized placebo-controlled phase III trial in Japan, South Korea and Taiwan is currently recruiting patients with unresectable HCC and will evaluate transcatheter arterial chemoembolization (TACE) in combination with either TSU-68 or placebo.

Cediranib (AZD2171) is another selective inhibitor of VEGFR-1, -2 and -3. Cediranib also exhibits activity against c-kit, PDGFR- $\beta$ , and FLT4 at nanomolar concentrations. In a phase II clinical study of advanced HCC, the median OS was 5.8 mo. No patients experienced confirmed response. The median time to progression was 2.8 mo<sup>[78]</sup>.

## EGFR, IGF AND HGF/c-MET SIGNALING

EGFR, a member of the human epidermal growth factor receptor (HER) family, contains an intracellular tyrosine kinase domain which can trigger signal transduction through the MAPK and PI3K/Akt/mTOR pathways. Thus, these receptors contribute to cell growth, differentiation, survival and adhesion<sup>[79]</sup>. EGFR overexpression has been reported in HCC. Immunohistochemical analysis by Buckley *et al*<sup>[80]</sup> revealed that EGFR was overexpressed in 50 (66%) of 76 HCCs, and fluorescence *in situ* hybridization (FISH) showed additional *EGFR* gene copies in 17 (45%) of 38 HCCs. EGFR-targeting drugs include anti-EGFR antibodies (such as cetuximab and panitumumab) and inhibitors of EGFR tyrosine kinases (such as erlotinib, lapatinib and gefitinib); these drugs have been used widely for the treatment of HCC.

Cetuximab is a recombinant chimeric monoclonal antibody that targets the extracellular domain of EGFR. In a phase II clinical trial of cetuximab in patients with advanced HCC, the median OS was 9.6 mo and the median PFS was 1.4 mo. The treatment was generally well tolerated. No treatment-related grade 4-5 toxicities occurred. Grade 3 aspartate aminotransferase, hypomagnesemia, and fever without neutropenia were each noted in 1 patient<sup>[81]</sup>. A randomized trial comparing gemcitabine-oxaliplatin (GEMOX) alone with a GEMOX-cetuximab combination is ongoing to define the real contribution of anti-EGFR therapy.

Erlotinib is a potent and reversible inhibitor of EGFR tyrosine kinase. In an *in vitro* study, erlotinib potentially suppressed the growth of human EGFR-expressing HCC cell lines. Erlotinib has been shown to inhibit the RAF/MEK/ERK signaling pathway and block signal

transducer and activator of transcription-mediated signaling<sup>[82]</sup>. A phase III placebo-controlled, double-blind SEARCH (Sorafenib and Erlotinib, a Randomized Trial Protocol for the Treatment of Patients with HCC) trial has been conducted in patients with advanced HCC. Three hundred sixty-two patients received sorafenib plus erlotinib and 358 received sorafenib plus placebo. No significant differences were observed in OS or TTP between the arms. Erlotinib, when added to sorafenib as the standard of care in advanced HCC, did not prolong overall survival<sup>[83]</sup>.

Lapatinib is a dual inhibitor of EGFR and HER-2/NEU that acts by docking into the ATP binding site of the two receptors<sup>[84]</sup>. Phase II results have indicated that lapatinib is well tolerated and have shown preliminary evidence of antitumor activity in HCC<sup>[85]</sup>. Among 40 patients with advanced HCC, the response rate was 5%, median PFS was 2.3 mo and median OS was 6.2 mo.

The IGF/IGFR signaling pathway regulates several cellular processes, including proliferation, motility and inhibition of apoptosis<sup>[86]</sup>. Ligand binding to IGF-1R triggers rapid receptor autophosphorylation, which in turn initiates downstream cellular effectors, ultimately leading to activation of PI3K, protein kinase B and the RAF/MEK/ERK pathway<sup>[87]</sup>. In HCC, dysregulation of IGF signaling occurs predominantly at the level of IGF-2. IGF-2 is overexpressed in 16%-40% of human HCCs, and IGF-2R (an alternative receptor for IGF-2) is underexpressed in approximately 80% of HCCs<sup>[88,89]</sup>. Associations have been reported between disease stage, metastasis and survival and the functions of IGF and IGFR in HCC<sup>[90,91]</sup>. Several strategies to target this system, including monoclonal antibodies against the IGF-1 receptor (IGF-1R) and small molecule inhibitors of the tyrosine kinase function of IGF-1R, are under active investigation.

Pre-clinical evidence obtained from HCC cells has shown that IMC-A12 (cituxumumab), a human monoclonal antibody that blocks IGF-1R. A phase I study of IMC-A12 yielded a partial response in HCC<sup>[92]</sup>. However, a subsequent phase II study in patients with advanced HCC showed that IMC-A12 is inactive as a monotherapy<sup>[93]</sup>. Up to 46% of the patients developed grade 3-4 hyperglycemia in this study. Hyperglycemia may be the dose limiting toxicity of IGF-1R monoclonal antibodies.

BIIB022 is an anti-IGF-1R monoclonal antibody that blocks binding of both IGF-1 and IGF-2 to IGF-1R. This agent does not appear to cause hyperglycemia, which is a common side effect of receptor-specific antibodies. A planned phase I / II study comparing sorafenib with or without BIIB022 in patients with advanced HCC was terminated due to a business decision by the sponsor company.

AVE1642 is another monoclonal antibody that specifically blocks IGF-1R signaling. This agent has been evaluated in combination with sorafenib in a phase I study in advanced HCC patients<sup>[94]</sup>. Long-lasting disease stabilization was observed in most patients with PD.

OSI-906 is a novel potent dual tyrosine kinase inhibitor of both IGF-1R and insulin receptor. The unique advantage of OSI-906 over the previous class of anti-IGF drugs is its ability to minimize IGF-2 activity in situations in which IGF-1R inhibition alone is not sufficient. The phase II study of second-line treatment for advanced HCC patients who failed first-line treatment with sorafenib (NCT01101906) was terminated because the sponsor decided not to pursue the development of this drug.

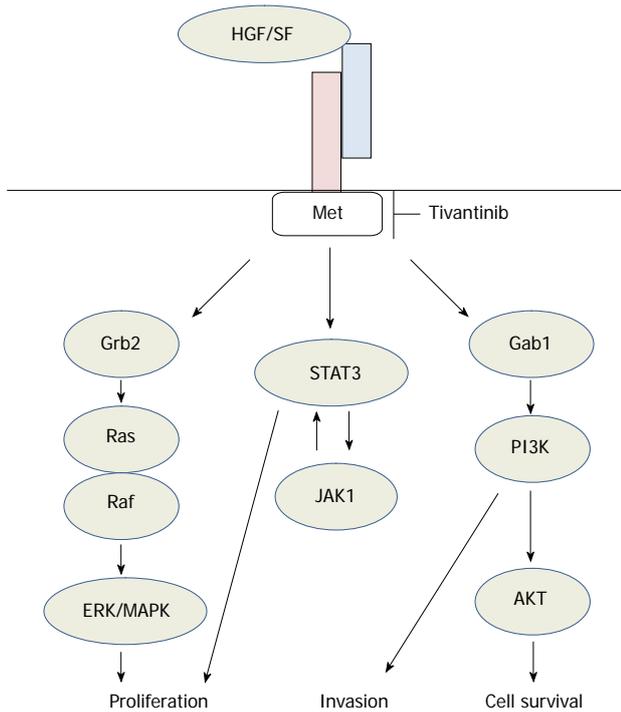
The HGF/Met pathway is involved in tumor growth, invasion and angiogenesis in various types of cancer<sup>[95]</sup>. c-Met is a tyrosine kinase receptor for the HGF ligand. HGF-induced activation of c-MET ultimately leads to the activation of downstream effector molecules, including phospholipase C, PI3K and ERK<sup>[96]</sup>. c-MET overexpression has been observed in 20%-48% of HCC, and overexpression has been linked with decreased 5-year survival in patients with HCC (Figure 3)<sup>[97-99]</sup>.

Tivantinib (ARQ 197) is a selective, oral MET receptor tyrosine kinase inhibitor with broad-spectrum antitumor activity as single agent. MET overexpression has been shown to be a negative prognostic factor in HCC after sorafenib failure. Tivantinib demonstrated a nearly doubling of PFS and OS in the MET high group compared to placebo in a phase II study as second-line treatment in patients with advanced HCC<sup>[100]</sup>. The activity of tivantinib in combination with sorafenib is also promising. Adverse events include hematological toxicity, asthenia and loss of appetite. The initially high incidence of neutropenia in patients with HCC led to dose reduction from 360 mg *bid* to 240 mg *bid*. Currently, a pivotal phase III study in advanced, MET-high HCC after sorafenib failure is planned.

## WNT-BETA-CATENIN PATHWAY

A major and early carcinogenic event in the development of HCC seems to be the abnormal regulation of the transcription factor  $\beta$ -catenin, a key component of the WNT signaling pathway.

During normal cell homeostasis, Wnt proteins are absent. Initiation of Wnt signaling leads to a series of events that cause loss of  $\beta$ -catenin phosphorylation, which prevents its degradation.  $\beta$ -catenin then accumulates in the cytoplasm and translocates into the nucleus. Hepatocytes with nuclear translocation of  $\beta$ -catenin display abnormal cellular proliferation and express membrane proteins involved in HCC, metastatic behavior, and cancer stem cells<sup>[101]</sup>. A high incidence of  $\beta$ -catenin mutations (nearly 40%) has been observed in HCC cases that occur in patients with HCV. HCC cases that occur in HBV patients display  $\beta$ -catenin activation that is induced in a mutation-dependent manner by the expression of HBx protein<sup>[102,103]</sup>. Agents targeting Wnt- $\beta$ -catenin are under development. Preliminary studies targeting the Wnt- $\beta$ -catenin pathway have demonstrated a potential space for new novel therapies to treat HCC.



**Figure 3** The c-Met signaling pathway suggested in hepatocellular carcinoma. Gab1: GRB2-associated binding protein 1; Grb2: Growth factor receptor-bound protein 2; HGF/SF: Hepatocyte growth factor/scatter factor; JAK1: Janus kinase 1; Met: Met proto-oncogene; PI3K: Phosphatidylinositol-3-kinase; STAT3: Signal transducer and activator of transcription 3; ERK: Extracellular signal-regulated kinase.

### JAK/STAT PATHWAY

The Jak/Stat pathway is activated by more than 40 cytokines and growth factors and is involved in multiple cell functions, including differentiation, proliferation, and apoptosis<sup>[104]</sup>. In this pathway, cytokines induce phosphorylation of the Janus tyrosine kinases (Jak1, 2 and 3 and Tyk2), which is followed by activation of Stat1-6<sup>[105]</sup>. The phosphorylation of Jak1, Jak2, and Tyk2 tyrosine kinases is not detected in normal livers but increases significantly between surrounding non-neoplastic liver and HCCs<sup>[106]</sup>. Activation of Stat1, Stat3, and Stat5 has been shown to be significantly higher in tumors than in the respective surrounding livers; pStat3 is higher in HCC with poor prognosis than in HCC with better prognosis<sup>[106]</sup>. The levels of Jak/Stat targets, including Bcl-xl, Mcl-1, cyclin D1, and c-Myc, are markedly elevated in the majority of HCCs. A phase I study of the JAK2 inhibitor AZD1480 in advanced solid malignancy (including HCC) is planned (NCT01219543).

### FUTURE PERSPECTIVES

Molecular targeted agents that have been introduced into clinical use in recent years have been approved for the treatment of a specific cancer and then frequently used to treat various other types of cancer (Table 1). Genetic alterations clearly play a major role in hepatocarcinogenesis, and abnormalities in several critical molecular

**Table 1** Molecular targets and therapeutic agents

Molecular targets	Therapeutic agents
VEGF/VEGFR	Sorafenib
	Bevacizumab
	Vatalanib (PTK787)
	Cediranib (AZD2171)
	Brivanib
EGF/EGFR	Sunitinib
	Linifanib (ABT869)
	Cetuximab
	Erlotinib
IGF/IGFR	Lapatinib
	OSI-906
	IMC-A12
Ras/Raf/MEK/ERK	AVE1642
	BIIB022
	Sorafenib
	Selumetinib (AZD6244)
PI3K/Akt/mTOR	AZD8055
	Everolimus
	Sirolimus
Wnt-β-catenin	Temsirolimus
	PFK118-310
	PFK115-584
MET	CGP049090
	Tivantinib

EGF: Epidermal growth factor; EGFR: EGF receptor; ERK: Extracellular signal-regulated kinase; FGFR: Fibroblast growth factor receptor; IGFR: Insulin-like growth factor (IGF) receptor; VEGF: Vascular endothelial growth factor; VEGFR: VEGF receptor; mTOR: Mammalian target of rapamycin; MET: Met proto-oncogene.

signaling pathways have been identified as contributing to tumor development and progression<sup>[107,108]</sup>.

Currently, sorafenib is the only effective systemic treatment option for advanced HCC. While the drug is effective for patients with advanced HCC, sorafenib prolongs life expectancy for only approximately three mo. To move beyond sorafenib monotherapy, a potential role for this agent in the adjuvant setting following surgical resection, radiofrequency ablation, or TACE or in combination with other targeted agents or chemotherapy is under investigation.

Several new promising multi-targeted molecules have been developed and are currently under investigation for the treatment of HCC (Table 2). Unfortunately, HCCs are refractory to many targeted therapies. Therefore, resistance to treatment remains the major challenge for targeted therapy. Many resistance mechanisms have been identified, including epigenetic changes, alternative splicing, target inactivation, upregulation of alternative pathways (by cellular adaptation to the pathway being targeted), and a range of mutations. A combination of different agents or a single “unspecific” inhibitor of several pathways may offer advantages to overcome resistance. Combinations of targeted agents with chemotherapy regimens also remain to be further explored. Molecular targeted therapy blocking angiogenesis has demonstrated somewhat promising results, but the efficacy of these agents is limited by survival pathways induced by hypoxia. Thus, the inhibition of hypoxia-induced survival signals

**Table 2** Efficacy results of targeted therapies for advanced hepatocellular carcinoma

Molecular targets/agents	Phase	Efficacy	Ref.
VEGF/VEGFR Sorafenib	Phase III SHARP Sorafenib <i>vs</i> placebo	Median OS: 10.7 mo <i>vs</i> 7.9 mo	[58]
Sunitinib	Phase III (Asian) Phase II	Median OS: 6.5 mo <i>vs</i> 4.2 mo Median PFS: 3.9 mo	[59] [65]
	Phase III Sunitinib <i>vs</i> sorafenib	Median OS: 9.8 mo Median OS: 7.9 mo <i>vs</i> 10.2 mo	
Brivanib	Phase II, first-line	Median PFS: 2.8 mo Median OS: 10 mo	[68]
	Phase II, second-line	Median PFS: 2.7 mo Median OS: 9.8 mo	[69]
	Phase III (BRISK-PS) Brivanib <i>vs</i> placebo	Median OS: 9.4 mo <i>vs</i> 8.3 mo TTP: 4.2 mo <i>vs</i> 2.7 mo RR: 12% <i>vs</i> 2%	[70]
	Phase III (BRISK-FL) Brivanib <i>vs</i> placebo	Median OS: 9.5 mo <i>vs</i> 9.9 mo TTP: 4.2 mo <i>vs</i> 4.1 mo RR: 12% <i>vs</i> 8%	[71]
Vatalanib (PTK787)	Phase I / II, combined with doxorubicin	OS: 7.3 mo PFS: 5.4 mo	[73]
Inifanib (ABT-869)	Phase II	TTP: 3.7 mo Median OS: 9.7 mo	[75]
Cediranib (AZD2171)	Phase II	Median OS: 5.8 mo TTP: 2.8 mo	[78]
EGF/EGFR Cetuximab	Phase II	Median OS: 9.6 mo Median PFS: 1.4 mo	[81]
Erlotinib	Phase III (SEARCH) Sorafenib/erlotinib <i>vs</i> orafenib/placebo	Median OS: 9.5 mo <i>vs</i> 8.5 mo TTP: 3.2 mo <i>vs</i> 4.0 mo	[83]
Lapatinib	Phase II	Median PFS: 2.3 mo Median OS: 6.2 mo	[85]
	Phase III Lipatinib <i>vs</i> sorafenib	Median OS: 9.1 mo <i>vs</i> 9.8 mo	
IGF/IGFR Cituxumumab (IMC-A12)	Phase II	Median OS: 8 mo	[93]
Ras/Raf/MEK/ERK Selumetinib (AZD6244)	Phase I / II	11 patients enrolled PR in 3, SD in 6, PD in 2 patients	[31]
PI3K/Akt/mTOR Everolimus	Phase I / II	Median PFS: 3.8 mo Median OS: 8.4 mo	[37]
Sirolimus	Phase II	Median PFS: 15.3 wk Median OS: 26.4 wk	[38]
MET Tivantinib	Randomized Phase II Tivantinib <i>vs</i> placebo ITT population c-Met high	Median TTP: 6.9 wk <i>vs</i> 6.0 wk Median OS: 6.6 mo <i>vs</i> 6.2 wk Median TTP: 11.7 wk <i>vs</i> 6.1 wk Median OS: 7.2 mo <i>vs</i> 3.8 wk	[100]

ITT: Intent to treat; OS: Overall survival; PD: Progressive disease; PFS: Progression-free survival; PR: Partial response; RR: Response rate; SD: Stable disease; TTP: Time to progression; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol-3-kinase; Met: Met proto-oncogene; EGFR: Epidermal growth factor (EGF) receptor.

might be required for targeted agents to block angiogenesis as an adjuvant therapy following TACE. Additionally, exploring potential markers that can help in identifying the patients who are most likely to respond (or to at least identify those who will not respond) to treatment is critical. Future development of genomic analysis of HCC will aid in the identification of specific biomarkers for patient selection for either single agent or combination molecular targeted therapies.

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## Endotherapy in chronic pancreatitis

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### Abstract

Chronic pancreatitis (CP) is a progressive disease with irreversible changes in the pancreas. Patients commonly present with pain and with exocrine or endocrine insufficiency. All therapeutic efforts in CP are directed towards relief of pain as well as the management of associated complications. Endoscopic therapy offers many advantages in patients with CP who present with ductal calculi, strictures, ductal leaks, pseudocyst or associated biliary strictures. Endotherapy offers a high rate of success with low morbidity in properly selected patients. The procedure can be repeated and failed endotherapy is not a hindrance to subsequent surgery. Endoscopic pancreatic sphincterotomy is helpful in patients with CP with minimal ductal changes while minor papilla sphincterotomy provides relief in patients with pancreas divisum and chronic pancreatitis. Extracorporeal shock wave lithotripsy is the standard of care in patients with large pancreatic ductal calculi. Long term follow up has shown pain relief in over 60% of patients. A transpapillary stent placed across the disruption provides relief in over 90% of patients with ductal leaks. Pancreatic ductal strictures are managed by single large bore stents. Multiple stents are placed for refractory

strictures. CP associated benign biliary strictures (BBS) are best treated with multiple plastic stents, as the response to a single plastic stent is poor. Covered self expanding metal stents are increasingly being used in the management of BBS though further long term studies are needed. Pseudocysts are best drained endoscopically with a success rate of 80%-95% at most centers. Endosonography (EUS) has added to the therapeutic armamentarium in the management of patients with CP. Drainage of pseudocysts, cannulation of inaccessible pancreatic ducts and celiac ganglion block in patients with intractable pain are all performed using EUS. Endotherapy should be offered as the first line of therapy in properly selected patients with CP who have failed to respond to medical therapy and require intervention.

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**Key words:** Chronic pancreatitis; Endoscopic retrograde cholangiopancreatography; Pancreatic sphincterotomy; Extracorporeal shockwave lithotripsy; Endosonography

**Core tip:** Chronic pancreatitis is a challenge to the therapeutic endoscopist. A patient with chronic pancreatitis can present with ductal calculi, leaks, pseudocysts, strictures, pancreatic malignancy or a biliary obstruction. Endoscopic therapy offers a high rate of success in properly selected patients. It offers many advantages over surgery, which for a long time was considered the gold standard in the treatment of chronic pancreatitis. This chapter deals with the management of chronic pancreatitis associated strictures, calculi, leaks and pseudocysts. The role of endosonography in management of pseudocysts, cannulation of inaccessible ducts and pain relief has also been discussed.

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## INTRODUCTION

Chronic pancreatitis (CP) is a disease of varied etiology and characterized by progressive and irreversible damage to the pancreas with resultant loss of both endocrine and exocrine functions. Alcohol, smoking, genetic factors and metabolic disorders are common etiological causes<sup>[1]</sup>. In our country the non alcoholic type of CP is more prevalent<sup>[2,3]</sup>. Irrespective of the etiology, the majority of patients with CP present with pain as the dominant symptom.

As the disease is irreversible, almost all therapeutic efforts are directed towards control of pain and management of complications associated with CP. For the therapeutic endoscopist, CP is a challenge as patients can present with ductal strictures, calculi, ductal disruption, pseudocysts, biliary strictures, duodenal narrowing or a pancreatic malignancy. Endotherapy is performed in patients with CP who are unlikely to respond or have failed medical therapy as well as to manage the above mentioned complications. Surgery has often been considered the best therapeutic option for patients with CP<sup>[4]</sup>. However with advances in technology and techniques, endotherapy is offered as first line management in many patients with CP. Endotherapy offers many distinct advantages over surgery. It has a high success rate and low morbidity in properly selected patients. The procedure can be repeated with no extra risk, unlike the morbidity and difficulty associated with repeat surgery. The results are comparable to surgery and failed endotherapy does not hinder subsequent surgery<sup>[5-8]</sup>. The endoscopic approach can also predict the response to surgical therapy<sup>[9]</sup>. The endoscopic techniques used are endoscopic retrograde cholangiopancreatography (ERCP) and endosonography (EUS). Extracorporeal shockwave lithotripsy (ESWL) is a part of the endoscopic armamentarium. Advances in EUS have improved therapeutic options, including pseudocyst drainage and cannulation of inaccessible main pancreatic duct (MPD).

In this review, we will discuss the role of endotherapy in diagnosis and management of CP related pancreatic ductal strictures, stones, common bile duct (CBD) strictures and pseudocyst.

## ROLE OF ENDOSCOPY IN THE DIAGNOSIS OF CP

ERCP was earlier used both for diagnosis and management of patients with CP. It has sensitivity of 73%-94% and specificity of 90%-100% in visualizing duct related changes in CP<sup>[10]</sup>. The emergence of magnetic resonance cholangiopancreatography (MRCP) with secretin stimulation, as well as EUS, has minimized the role of ERCP in diagnosing CP. EUS is a better diagnostic modality, especially in early and less advanced CP, as it identifies both ductal and parenchymal changes<sup>[11]</sup>. EUS has a sensitivity of close to 100% as compared to 80% with ERCP in patients with early CP<sup>[12]</sup>. MRCP being non-invasive offers a better alternative to ERCP for visualizing ductal changes.

## CP WITH MINIMAL DUCTAL CHANGES

Painful CP can occasionally present with minimal or no ductal change in absence of ductal strictures or stones. This is classified as type I CP according to Cremer classification or mild CP of Cambridge classification<sup>[13,14]</sup>. Endoscopic pancreatic sphincterotomy (EPS) is a documented mode of therapy and offers symptomatic relief in some of these patients. Both the standard pull type and the needle knife sphincterotomy over a stent can be performed. A 64% relief in pain on follow up of 6.5 years has been reported following EPS<sup>[15]</sup>. High success rates of 98% and low complication rates of around 4% have been reported on retrospective analysis<sup>[16]</sup>. Randomized studies have shown a higher incidence of pancreatitis in high risk patients following pull type sphincterotomy as compared to the needle knife technique<sup>[17]</sup>. Most workers report an incidence of around 12% for post EPS pancreatitis. Placement of a nasopancreatic tube (NPT) or pancreatic stent can reduce this incidence significantly<sup>[18]</sup>. Restenosis is reported in around 14% of patients on long term follow up<sup>[19]</sup>. It is believed that restenosis is less common following the longer incision with the pull type as compared to needle knife technique<sup>[20]</sup>. The presence of periductal fibrosis seen in patients with CP may lower the incidence of post procedure pancreatitis. An additional biliary sphincterotomy is only indicated in the following conditions<sup>[21]</sup>: (1) presence of cholangitis; (2) CBD > 12 mm diameter; (3) serum alkaline phosphates > 2 times upper limit of normal; and (4) difficult access to MPD.

### Minor papilla sphincterotomy

Minor papilla sphincterotomy (MiES) was first performed by Cotton<sup>[22]</sup>. It is indicated in those patients with CP with minimal ductal changes who have a pancreas divisum or a dominant dorsal duct. Both the pull type and needle knife technique can be used. The evidence of any definite benefit from MiES is debatable as studies include small numbers of heterogeneous patients and are not conclusive. Significant pain relief on a 2-year follow up has been reported following MiES and stenting of patients with CP<sup>[23]</sup>. Relief of pain is also seen in 41% of patients with CP following MiES as compared to 77% with acute recurrent pancreatitis or 33% of patients with CP with no pain<sup>[24]</sup>. Post ERCP pancreatitis has been reported in up to 15% of patients<sup>[25]</sup> and restenosis was seen in 20%-24% on a 6-year follow up<sup>[26]</sup>.

## ENDOSCOPIC MANAGEMENT OF PANCREATIC DUCTAL STRICTURES

Strictures of MPD are frequently seen as a consequence of CP and could be due to inflammation or fibrosis. In our experience of 1000 patients who underwent ESWL, the incidence of strictures was 18%<sup>[2]</sup>. MPD strictures are defined as a high grade narrowing of MPD with one of the following<sup>[19,27]</sup>: (1) MPD dilatation > 6 mm beyond

the stricture; (2) failure of contrast to flow alongside the stricture or 6 Fr NPT; and (3) presence of pain during continuous perfusion of the NPT with normal saline for 24 h.

Endotherapy is ideal for single strictures in the head while isolated strictures in the tail or multiple strictures with a chain of lake appearance are not amenable to endotherapy<sup>[9]</sup>. Prior to stent placement tight strictures need to be dilated with Teflon bougies, Sohendra stent retriever or a balloon dilator<sup>[9,27]</sup>. Large bore stents 7-10 Fr should be deployed as they have longer patency<sup>[27]</sup>. Delhaye *et al*<sup>[27]</sup> followed a protocol where a single stent was placed across a stricture and exchanged every 6 mo or when the patient was symptomatic. Stents were placed for 24 mo. Patients were restented if symptoms recurred. Surgery was considered if patients responded to stent placement but needed frequent or repeated stenting. Cumulative data from several workers revealed pain relief between 70%-94% for a single pancreatic stent on follow up of 14-69 mo<sup>[9]</sup>. Recurrence of strictures was reported in 38% of patients after 2 years follow up<sup>[28]</sup>. The concept of multiple plastic stenting for MPD strictures not responding to a single stent placement was advocated by Costamagna *et al*<sup>[29]</sup>. In their study, after removal of a single stent, the stricture was dilated and multiple plastic stents 8.5-11.5 Fr diameter were placed. A mean of 3 stents were used. The stents were removed 12 mo later. Stricture resolution was seen in 95% and pain relief in 84% on a 38 mo follow up.

Complications with pancreatic stenting can occur. Occlusion was seen with the passage of time and migration was present in 10% of patients<sup>[30]</sup>. Distal migration and impaction on the opposite duodenal wall can cause perforation while proximal migration into the pancreas is a technical challenge for the endoscopist. The possibility of stent induced fibrosis has raised concerns<sup>[31]</sup>. However with the preexisting fibrosis of MPD there has been no significant clinical impact. The search for an ideal pancreatic stent continues and a new "wing stent" to prevent clogging as well as an "S" shaped stent to prevent migration are undergoing evaluation<sup>[32,33]</sup>. The use of covered metal stents (CSEMS) for pancreatic strictures is also under evaluation. The initially used CSEMS had the disadvantage of stent migration. Subsequently a new "bumpy stent" has been tried for MPD strictures in 32 patients<sup>[34,35]</sup>. The stent had antimigratory properties and its contours adapted to the MPD. These were extracted at 3 mo and were effective in resolving the MPD strictures. However they were associated with the formation of de novo strictures and further trials are needed to evaluate their long term efficacy and safety.

European Society of Gastrointestinal Endoscopy (ESGE) guidelines state that dominant PD strictures be treated by placing a single 10 Fr stent with stent exchanges planned for 1 year. Multiple plastic stents should be deployed in a stricture which persists after 1 year of single stent placement<sup>[36]</sup>. Uncovered SEMs should not be placed in MPD. ESGE guidelines also state that tem-

porary placement of fully covered SEMs should only be performed in the setting of trials<sup>[36]</sup>.

## ENDOTHERAPY OF PANCREATIC DUCTAL CALCULI

Pancreatic ductal calculi are a consequence of CP and tend to aggravate or produce pain by obstructing pancreatic ducts and producing upstream hypertension. They can occur in 50% of patients with CP<sup>[8]</sup>. Stones seen in the tropics and of the non-alcoholic type of CP tend to be larger and denser than those seen in the alcoholic variety<sup>[37,38]</sup>. The large size could also be due to delay in reporting for therapy<sup>[2]</sup>. Stones > 5 mm in size can usually be extracted with a Dormia basket, or balloon trawl following EPS. However stones > 5 mm in size are often impacted and difficult to extract by the standard techniques<sup>[2,37,39]</sup>. Large calculi need to be fragmented prior to extraction or spontaneous expulsion from the MPD. ESWL is now accepted as the standard of care in the management of large PD calculi not amenable to routine endotherapy<sup>[2,36,37,40-45]</sup>. ESWL is very effective in fragmenting both radio-opaque and radio-lucent calculi in the MPD. A meta-analysis of 17 studies with a total of 491 patients revealed a clearance rate between 37%-100% and good pain relief<sup>[46]</sup>. Another review of 11 studies with over 1100 patients showed successful stone fragmentation in 89%<sup>[47]</sup>. Our own single center study of over 1000 patients shows complete clearance in 76% patients and partial clearance in another 17% patients following ESWL and endotherapy for large calculi<sup>[2]</sup> (Figures 1 and 2).

The following protocol is followed at our center for patients with large PD calculi<sup>[2]</sup>. Patients with large calculi in the head or body and with pain as the main complaint are subjected to ESWL. Patients with isolated calculi in the tail, multiple MPD strictures, extensive calculi in head, body and tail, associated head mass, pseudocysts and pregnancy are excluded from ESWL. The procedure is performed with a III generation electromagnetic lithotripter with bi-dimensional fluoroscopy and ultrasound targeting facility. (Delta compact-Dornier MedTech Wessling Germany). Epidural anesthesia is preferred in most patients<sup>[48]</sup>. It is effective and offers many advantages as reported in our study of over 1500 patients. Radio-opaque calculi are subjected to ESWL under fluoroscopic guidance. For radio-lucent calculi, a NPT is placed and contrast is passed through this tube to help localize the calculi. The aim of fragmentation is to break the calculi to 3 mm or less to facilitate their extraction or expulsion<sup>[2,49]</sup>. An average of 3 sessions is generally required (5000-6000 shocks per session). The protocol is shown in Figure 3. A few studies have advocated use of ESWL alone followed by spontaneous expulsion of fragments<sup>[50]</sup>. A randomized controlled trial of 55 patients compared results of ESWL and ERCP with ESWL alone. The only difference was higher cost and longer stay in the ESWL and ERCP group<sup>[51]</sup>. At our center, we prefer to extract

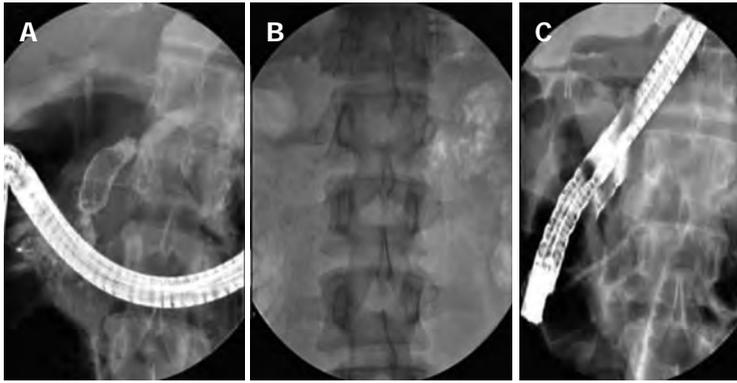


Figure 1 Large pancreatic calculi in head (A), genu in a patient with pancreas divisum (B) and chronic pancreatitis cleared by extracorporeal shockwave lithotripsy followed by pancreatic stenting<sup>[49]</sup> (C).

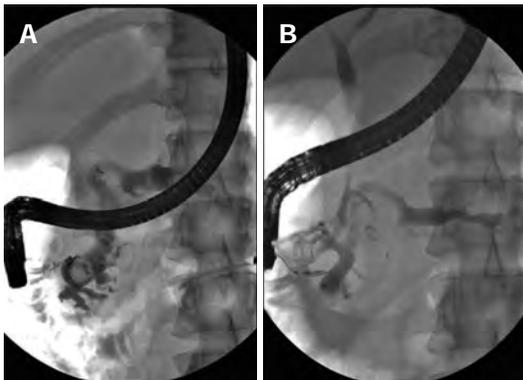


Figure 2 Large pancreatic calculi in head. Post extracorporeal shockwave lithotripsy reduction in diameter of main pancreatic duct<sup>[49]</sup> (A, B).

the fragments from the MPD by ERCP following the ESWL procedure as fragments tend to be denser and adherent and do not clear spontaneously<sup>[2,49]</sup>.

Short term pain relief following ESWL was seen in 84% of our patients<sup>[2]</sup> and similar results have been reported by others<sup>[39]</sup>. Very few long term follow up studies are available. Two-thirds of patients were found to be pain free on long term follow up<sup>[8,52]</sup>. A recent study showed pain relief in 85%, complete pain relief with no narcotic use in 50% and avoidance of surgery in 84% of 120 patients on long term follow up after ESWL<sup>[53]</sup>. Our own data on long term follow up is encouraging and over 60% of patients are pain free on follow up of more than 5 years<sup>[54]</sup>. In conclusion, in properly selected group of patients with large PD calculi, ESWL is a useful tool and provides adequate long term pain relief. A few patients also benefit in exocrine and endocrine dysfunction though the numbers are too small to be significant<sup>[54]</sup>. ESWL is a safe procedure and well tolerated. Minor side effects such as transient pain and bruising of skin at the site of shock delivery have been described<sup>[2,37,49]</sup>. The incidence of pancreatitis is not higher following ESWL.

Other techniques for extraction of large PD calculi include intraductal laser or electro hydraulic lithotripsy through a pancreatoscope or spyscope<sup>[55,56]</sup>. Experience with these modalities is small and success rates are discordant. These procedures are technically difficult and require non standard equipment. At present, they are only

to be considered as second line management after failed ESWL<sup>[36]</sup>.

## CHRONIC PANCREATITIS RELATED BENIGN BILIARY STRICTURES

CBD strictures occur in 3%-46% of patients with CP<sup>[30]</sup>. Strictures can be reversible due to inflammation or compression with a pseudocyst. They are irreversible following fibrosis. ESGE guidelines recommend treating CP related benign biliary strictures (BBS) in cases with symptoms, secondary biliary cirrhosis, biliary stones, asymptomatic elevation of serum alkaline phosphates > 2-3 times upper limit of normal or raised serum bilirubin persisting for over 1 mo<sup>[36]</sup>. Placement of a single plastic stent in the CBD is associated with poor success rates. Long term results have disappointing and sustained benefit is seen in around 25% of patients on follow up of 46 mo<sup>[57]</sup>. Single plastic stents are associated with poor resolution and higher relapse rate. The presence of pancreatic head calcification is an important factor for failure of this therapy<sup>[58]</sup>. Placement of multiple plastic stents in CP related BBS is technically successful in over 95% of patients and offers the best results. Complete therapy requires approximately four ERCP procedures and stents exchanges performed every 3 mo for 1 year. Single stents provided relief in 31% of 350 patients as compared to 62% in 50 patients who received multiple stents<sup>[36]</sup>. Catalano *et al*<sup>[59]</sup> performed a non-randomized study comparing single and multiple plastic stents in CP related BBS. Clinically, success was reported in 92% with multiple stents as compared to 24% with single stents. Uncovered SEMs for BBS are not advocated and partially or fully covered SEMs have been used with a success rate of 50%-80% on follow up for 22-28 mo<sup>[60,61]</sup>. A recently conducted multicenter trial using fully covered SEMs (FCSEMS) in BBS included 127 patients of CP. It concluded that FCSEMS may be useful for treatment of BBS particularly in patients with CP<sup>[62]</sup>. There has been no head to head study comparing single or multiple plastic stents and metal stents in BBS due to CP and surgery. The choice and option of surgery depends upon patient preference, expertise at the treating center and the presence of co morbidities such as cirrhosis or collaterals.

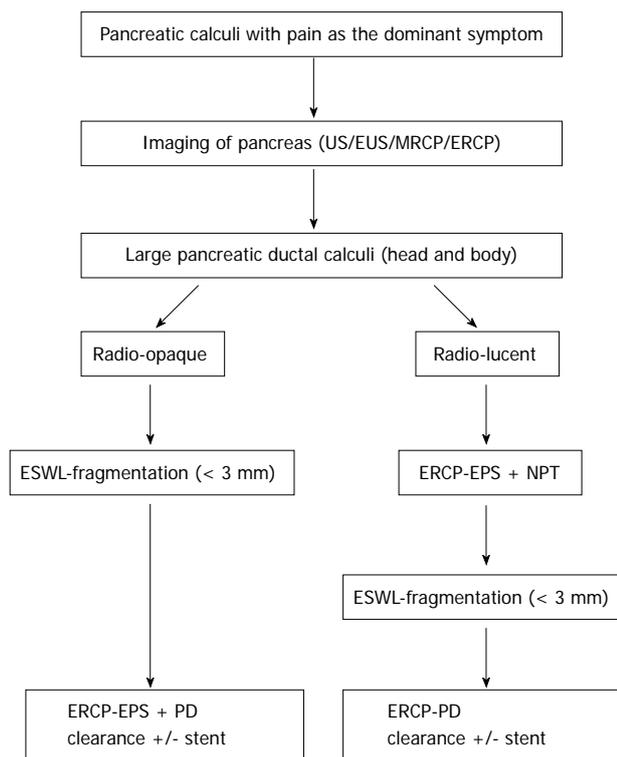


Figure 3 Protocol followed for extracorporeal shockwave lithotripsy in chronic calcific pancreatitis. NPT: Naso pancreatic tube; EPS: Endoscopic pancreatic sphincterotomy; ERCP: Endoscopic retrograde cholangiopancreatography; ESWL: Extracorporeal shockwave lithotripsy; US: Ultrasonography; PD: Pancreatic duct; EUS: Endosonography; MRCP: Magnetic resonance cholangiopancreatography.

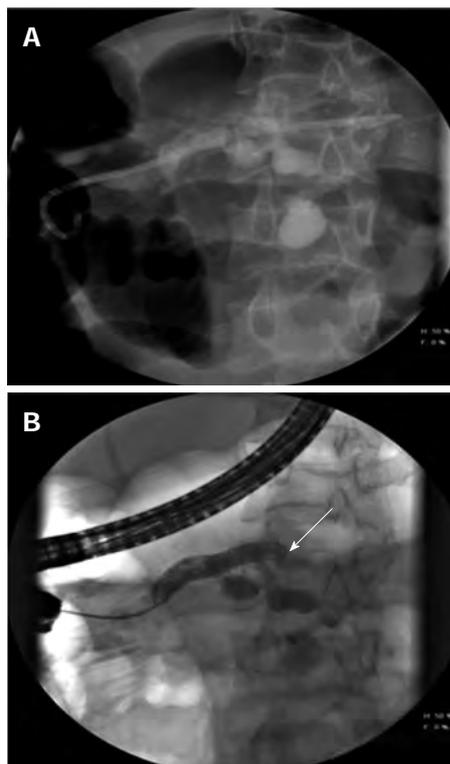


Figure 4 Mid body leak (arrow) with extravasated contrast in a patient with chronic pancreatitis (A) and dilated pancreatic duct (B). Stent placed across the leak.

## PANCREATIC DUCTAL LEAKS

Leaks from the MPD or side branches can occur following blow out of the ducts due to obstruction by stone or strictures. PD leak is defined as extravasation of contrast material from the ductal system at ERCP<sup>[63]</sup>. Disruption may be partial or complete and leads to fluid collection, pseudocysts, ascites, pleural effusion and external or internal fistulas<sup>[9,27]</sup>. Placement of transpapillary stents offers the best treatment in patients with PD disruption as it converts the high pressure ductal system into a low pressure one with preferential flow across the stents<sup>[27]</sup>. Resolution of leak was seen in 92% of patients when the stent bridged the disruption, 50% when placed proximal to the disruption and 44% when a short transpapillary stent was placed<sup>[63]</sup> (Figure 4). In patients with complete transection where stenting is not feasible a multidisciplinary approach with a help of interventional radiologist or the surgeon may be required.

## ENDOSCOPY OF PSEUDOCYSTS

Pancreatic pseudocyst (PPC) in CP is the result of disruption of the MPD or its side branches and occurs in 20%-40% of patients<sup>[64]</sup>. Disruption generally follows obstruction by stones or strictures. Treatment is indicated for symptomatic PPC or those which increase in size.

Symptoms result due to compression of adjacent structures or due to infection. It has also been suggested that prophylactic treatment be performed in certain specific situations to prevent complications. These include Pancreatic-pleural fistula, cysts > 5 mm lasting for over 6 wk, compression of major vessels or presence of large pancreatic stones in MPD<sup>[65]</sup>. There is generally a low rate of spontaneous resolution of PPC in patients with CP though small asymptomatic cysts can be followed up<sup>[66]</sup>. Drainage of pseudocysts can be transmural (transgastric or transduodenal) or transpapillary. Transmural drainage is ideal for PPC which bulge into the lumen of stomach or duodenum. Transduodenal drainage offers the best success when compared to transgastric drainage<sup>[67]</sup>. This is because cystoduodenal fistulas tend to remain patent longer than cystogastric fistulas. Placement of one or more pig tailed stents is better when compared to straight stents. Straight stents are associated with a higher rate of bleed (around 7%) as well as migration<sup>[68]</sup>. Stents should be left in place for a longer duration as their removal within 2 mo is associated with a higher incidence of PPC recurrence<sup>[36]</sup>. Pseudoaneurysm can complicate management of PPC because of the associated haemorrhage and consequent high mortality<sup>[69]</sup>. Delhaye *et al*<sup>[27]</sup> recommend prophylactic embolization of pseudoaneurysms prior to drainage of an adjacent PPC.

Transpapillary drainage is reserved for small cysts (< 6 cm size) and those in communication with the MPD. The role of EUS guided drainage for nonbulging PPC

will be discussed in the next section. Comparison of EUS guided drainage with surgery in an RCT revealed that endoscopic drainage was significantly better than surgery in terms of cost and length of stay over a 3 mo follow up<sup>[70]</sup>. Complications include bleed, infection and leak of around 4% each with a mortality of 0.5%<sup>[71]</sup>. Infection is more likely with transpapillary drainage and leak is more likely with transmural drainage. Routine antibiotic administration is recommended for drainage of PPC<sup>[72]</sup>. With a success rate of 80%-95% at most centers, a recurrence rate of 10%-20% and results comparative or better than surgery, endoscopy is the preferred first line of management for patients with PPC in the background of CP<sup>[27,36]</sup>.

## ENDOSONOGRAPHY IN CP

EUS is an excellent diagnostic modality especially in patients with early CP. It also has a definite therapeutic role in the following situations and these are discussed briefly.

### PPC drainage

EUS is ideal for drainage of nonbulging PPC and cysts as far as 4 cm from the stomach or duodenal wall have been drained<sup>[73]</sup>. Around 44%-53% of PPCs belong to this category. In the presence of collaterals the site of drainage is better identified with EUS, thus making the procedure safer. The complication rate is however similar when PPCs are drained with or without EUS guidance<sup>[74]</sup>. A recent randomized trial comparing EUS guided and surgical cystogastrostomy for pseudocysts revealed shorter hospital stay, lower cost and better physical and mental health in the endoscopy group. None in the endoscopy group had pseudocyst recurrence and therapy was successful in all the patients<sup>[75]</sup>.

### EUS guided access of MPD

EUS guided access or drainage is indicated following failed conventional drainage of MPD. It can be *via* the stomach (pancreatogastric) or duodenum (pancreatobulbar). The duodenal route is preferred because of better stability of the EUS scope<sup>[9]</sup>. A guidewire can be passed into the duodenum for a rendezvous procedure or transmural drainage can be performed. Success rates of 77%-92% have been reported<sup>[76,77]</sup>. Complications include pain, bleeding, perforation and hematoma and morbidity of 0%-44% has been reported<sup>[76-78]</sup>. EUS guided access of the MPD is a technically challenging procedure and should always be performed by experts and under radiological guidance<sup>[9]</sup>.

### EUS guided celiac block

Patients who have failed to respond to intensive medical or endoscopic therapy and are not candidates suitable for surgery can be provided relief from pain by EUS guided celiac block. A combination of corticosteroids (triamcinolone) and anesthetic agents (bupivacaine) is injected in and around the celiac plexus under EUS guidance. A recent meta analysis has reported pain relief in 50%-55%

of patients though the pain relief is transient<sup>[79,80]</sup>. Patients who are younger than 45 years or have previous pancreatic surgery are less likely to benefit<sup>[81]</sup>. EUS guided celiac block is shown to be superior to fluoroscopy guided celiac block for pain relief and pain preference in our study<sup>[82]</sup>. EUS guided nerve block can produce diarrhea, hypertension due to sympathetic blockade and unopposed parasympathetic activity<sup>[11,80]</sup>.

## CONCLUSION

In conclusion, management of CP is a multidisciplinary task involving the physician, endoscopist, interventional radiologist and surgeon. Their roles are complementary to each other. As mentioned earlier endotherapy is effective, less invasive than surgery, offers good results and is associated with low morbidity and mortality. It can be repeated and does not interfere with any subsequent surgical procedure. It is therefore advisable to offer endotherapy as the first line treatment in properly selected patients with CP.

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## HER2 therapies and gastric cancer: A step forward

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**Core tip:** Approaches for treatment advanced gastric cancer are object of interesting debates toward scientific community worldwide over the last 20 years. Chemotherapy based on platinum and fluoropyrimidine agents remained up to now the standard of care for those patients, otherwise triplet therapy either an anthracycline or taxane may be considered. Herein we provide an additional discussion regarding the role of biologic agents, such as trastuzumab and novel therapies for improve survival in this field.

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### Abstract

Gastric cancer usually is diagnosed in advanced stages and thus current medical practice affords limited therapeutic options. However, recent studies established the role of human epidermal growth factor receptor 2 (HER2) in clinical management. Trastuzumab, an anti-HER2 monoclonal antibody, acquired a main role in advanced gastric cancer harboring HER2 overexpression and/or amplification improving survival to 17.1 mo according to trastuzumab for gastric cancer phase III trial results. Also, new promising drugs, such as c-Met inhibitors, are in development and assessment for this setting. Certainly, novel drugs will emerge in the next few years for help oncologists improve clinical management of advanced gastric cancer providing higher survival and quality of life. In this mini-review we will discuss some issues in this regard and provide an actual overview of this setting.

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**Key words:** Gastric cancer; Trastuzumab; c-Met; Human

### INTRODUCTION

Gastric cancer (GC) is one of the leading types of cancer worldwide. Although the trend in death rates<sup>[1]</sup> for GC is decreasing, this tumor continues to have a poor prognosis and few efficacious therapeutic options particularly in advanced stages. Since most of symptoms for this type of cancer are nonspecific and screening strategies in many countries are absence, GC is usually diagnosed in advanced stages. The predominant histological type of GC is adenocarcinoma (95% of tumors) and the main adenocarcinomas sub-types are intestinal, diffuse and mixed type. Recent studies showed the human cancer is the human epidermal growth factor receptor in advanced GC personalizing treatment<sup>[2-5]</sup>. Herein we will discuss issues concerning novel biologic agents for advanced gastric cancer, focusing in anti-human epidermal growth factor receptor 2 (HER2) therapies, such as trastuzumab, and promising novel agents.

## HER2 AND GASTRIC CANCER

Treatment depends on the site and extent of the tumor<sup>[4,6,7]</sup>. Treatment objectives vary from through curative approaches, such as curative surgery, radiotherapy and perioperative chemotherapy, that may improve the survival rate of operable GC patients; to palliative approaches in advanced stage patients or those who are subject to relapse after prior curative surgery<sup>[7,8]</sup>. For advanced patients, 5-fluorouracil (5-FU) plus platinum remain standard treatment regimens, with or without an anthracycline or taxane<sup>[9]</sup>. This therapeutic regimen offers a response rate of 30%-50% with 9-11 mo median overall survival (OS)<sup>[10]</sup>. Given these poor results, an investment in new treatment weapons is required. One of the most considerable innovative targets in human cancer is the human epidermal growth factor receptor (EGFR) family<sup>[11]</sup>. The human HER family includes four structurally related members, HER1 (ErbB1, also known as EGFR), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4)<sup>[12]</sup>. Relatively to HER2, this is highly expressed in a significant proportion of GC<sup>[13]</sup> and thus it is nowadays considered an excellent therapeutic target. GC harboring HER2 overexpression was shown to have a worse prognosis<sup>[14]</sup>. In HER2-amplified patients the median survival was 5.5 mo compared with 12.6 mo in non-amplified patients. HER2 overexpression was more commonly seen in the intestinal-type than diffuse-type cancers (32% *vs* 6%)<sup>[15-17]</sup>.

## HER2 MOLECULAR TESTS AND TRASTUZUMAB

HER2 overexpression can be determined by immunohistochemistry (IHC) using a monoclonal antibody or by the detection of HER2 gene amplification through fluorescent *in situ* hybridization (FISH)<sup>[18-20]</sup>. Thus, it is current practice to test all new diagnoses of GC for HER2 by IHC<sup>[21,22]</sup>. Tumors can be classified by IHC as IHC 0/1+, negative resulted; IHC2+, equivocal resulted and it is recommended FISH testing, and IHC3+, positive resulted<sup>[18,23]</sup>.

In the trastuzumab for gastric cancer (ToGA) trial<sup>[2]</sup>, trastuzumab, a recombinant humanized monoclonal antibody that targets the extracellular domain IV of the HER2 protein, was evaluated in HER2 overexpressing gastric and gastroesophageal junction (GEJC) cancer. In the mentioned study, patients with GC or GEJ that showed HER2 overexpression were eligible for the analysis and randomized in two arms. To one arm standard chemotherapy alone (5-FU/capecitabine plus cisplatin) was administered while to the other arm it was administered chemotherapy plus trastuzumab. Median OS was 13.8 mo in those assigned to trastuzumab plus chemotherapy compared with 11.1 mo in those assigned to chemotherapy alone<sup>[24]</sup>. The median of progression-free survival (PFS) was increased with the addition of trastuzumab to standard chemotherapy: 6.7 mo in the trastuzumab arm and 5.5 mo in the chemotherapy alone

arm. The overall response rate was 47.3% *vs* 34.5% in trastuzumab plus chemotherapy and chemotherapy, respectively. The toxicity did not increased substantially with trastuzumab addition; however, the most common grade 3/4 adverse reactions associated with trastuzumab in metastatic GC were neutropenia, diarrhea, fatigue, anemia, stomatitis, weight loss, upper respiratory tract infections, fever, thrombocytopenia, mucosal inflammation, nasopharyngitis and dysgeusia. Thus, the ToGA trial showed that trastuzumab in combination with chemotherapy can be considered as a new standard option for patients with HER2-positive advanced GC or GEJC. So, trastuzumab was approved by the Food and Drug Administration and the European Medicines Agency (EMA) for patients with HER2-positive metastatic GC or GEJ who have not received previous anticancer therapy for metastatic disease.

## NOVEL AGENTS AND PROMISING MOLECULES

Nevertheless, others monoclonal antibodies have been developed as an alternative to trastuzumab<sup>[25-28]</sup>. For example, HER dimerization inhibitor, such as pertuzumab, which in combination with the trastuzumab has shown to have a promising effect in experimental models of GC<sup>[29,30]</sup>. In addition, some studies with anti-HER2 combination treatments indicate that the use of more than one HER2-targeted therapy was superior to one of these agents alone, particularly in breast cancer (BC) HER2 positive<sup>[31-33]</sup>. For instance, the CLEOPATRA<sup>[34]</sup> phase III trial compared the efficacy and safety of pertuzumab, trastuzumab, and docetaxel with placebo, trastuzumab, and docetaxel in patients with HER2-positive first-line metastatic breast cancer, showed a significant improvement in OS with addition of pertuzumab. So, there is need for planning studies to assess the safety and efficacy of the pertuzumab in the GC HER2 positive<sup>[35,36]</sup>.

However, when the patients acquire resistance to trastuzumab, what to do? The molecular mechanisms underlying trastuzumab resistance in GC are still unknown, but intra-tumoral heterogeneity of this tumor may contribute to this resistance<sup>[12,37-39]</sup>. There are some mechanist theories in a study that attempted to explain this phenomenon, *e.g.*, that catecholamine-induced  $\beta$ 2-AR activation mediates desensitization of GC cells to trastuzumab through up regulation of the MUC4 expression<sup>[40,41]</sup>, or that interaction between HER2 and insulin-like growth factor 1 receptor in trastuzumab-resistant breast cancer cells and involved in cross-talk that results in p27 downregulation<sup>[42]</sup>. Furthermore, hepatocyte growth factor (HGF) and its receptor, the trans-membrane tyrosine kinase c-Met, promote cell proliferation, survival, motility and invasion as well as morphologic changes that stimulate tissue repair and regeneration in normal cells but can be co-opted during tumor growth<sup>[28]</sup>. Previous studies reported that high levels of HGF or c-Met are associated with poor prognosis in gastric can-

cer, due to gene amplification and protein overexpression of c-Met drive resistance to epidermal growth factor receptor family inhibitors, both in preclinical models and in patients<sup>[21,27,28,43,44]</sup>. Only a few phase I - II trials<sup>[26,45]</sup> recently assessed the role of c-Met inhibitors, such as crizotinib<sup>[46]</sup> and foretinib<sup>[26]</sup>, in gastric cancer setting. In a study by Lennerz *et al.*<sup>[46]</sup> two patients harboring MET amplification were treated with crizotinib and presented tumor shrinkage (-30% and -16%) and experienced progression after 3.7 and 3.5 mo. Shah *et al.*<sup>[26]</sup> reported 67 advanced gastric cancer patients who were treated with foretinib irrespective of c-Met status. Best response was stable disease (SD) in 10 (23%) patients receiving intermittent dosing and 5 (20%) receiving daily dosing; SD duration was 1.9-7.2 mo (median 3.2 mo). Of 67 patients with tumor samples, 3 had MET amplification, one of whom had SD. Treatment-related toxicity occurred in 91% of patients<sup>[26]</sup>. Thus, the response to this dilemma is not so simple and current there are many options for explore in this regard.

In this regard, others classes of targeted drugs, including tyrosine kinase inhibitors, such as lapatinib<sup>[47]</sup> and dacomitinib<sup>[48]</sup>, mammalian target of rapamycin pathway inhibitors, such as everolimus<sup>[49]</sup>, have also been investigated. Lapatinib inhibits the catalytic activity of the EGFR and it is also a HER2 inhibitor; thus, it is a dual tyrosine kinase inhibitor of both EGFR and HER2. The SWOG S413 trial<sup>[47]</sup> analyzed lapatinib in the first line therapy in patients with advanced or metastatic GC showing 9% response rate (11% overall response rate) and a median OS of 4.8 mo. In summary, lapatinib as a single agent presents reduced responses, but in combination with other chemotherapeutic agents may have additional benefits. Dacomitinib<sup>[18]</sup> is a pan-HER inhibitor with potential use in cancer treatment via mutations or overexpression/amplification of HER family members or their target molecules alone or in combination with chemotherapeutic and/or molecular-targeted agents, however, there are no clinical trials phase II / III to justify its use in GC patients.

## CONCLUSION

Nowadays, an interesting biologic option is available, such as trastuzumab, for combination with platinum-5-FU for prolongs OS in a sub-set of patients. However, only 20% of advanced GC harbor with HER2 overexpression and thus a large number of patients will not acquire benefit from this innovative option. Thus, further alternatives are warranted for overcome this issue. Others biological agents are under investigation, but without immediate results for the current clinical practice. Crizotinib, foretinib and pertuzumab seems to be promising due to preliminaries small studies. However, results from larges phase III trials are still need to determine whether those innovative agents would be place in the current scenario. In conclusion, HER2 targeted therapy is responsible for a significant increase in survival of patients with GC in

advanced stages. Unfortunately, the GC continues to still have a poor prognosis. In the future it is intended to develop new trials and look for other genetic alterations that may be highly specific therapeutic targets and less toxic as well.

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## Alteration in gene expression profile and oncogenicity of esophageal squamous cell carcinoma by *RIZ1* upregulation

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### Abstract

**AIM:** To investigate the effect of retinoblastoma protein-interacting zinc finger gene 1 (*RIZ1*) upregulation in gene expression profile and oncogenicity of human esophageal squamous cell carcinoma (ESCC) cell line TE13.

**METHODS:** TE13 cells were transfected with pcDNA3.1(+)/*RIZ1* and pcDNA3.1(+). Changes in gene expression profile were screened and the microarray results were confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Nude mice were inoculated with TE13 cells to establish ESCC xenografts. After two weeks, the inoculated mice were randomly divided into three groups. Tumors were injected with normal saline, transfection reagent pcDNA3.1(+) and transfection reagent pcDNA3.1(+)/*RIZ1*, respectively.

Tumor development was quantified, and changes in gene expression of *RIZ1* transfected tumors were detected by RT-PCR and Western blotting.

**RESULTS:** DNA microarray data showed that *RIZ1* transfection induced widespread changes in gene expression profile of cell line TE13, with 960 genes upregulated and 1163 downregulated. Treatment of tumor xenografts with *RIZ1* recombinant plasmid significantly inhibited tumor growth, decreased tumor size, and increased expression of *RIZ1* mRNA compared to control groups. The changes in gene expression profile were also observed *in vivo* after *RIZ1* transfection. Most of the differentially expressed genes were associated with cell development, supervision of viral replication, lymphocyte costimulatory and immune system development in esophageal cells. *RIZ1* gene may be involved in multiple cancer pathways, such as cytokine receptor interaction and transforming growth factor beta signaling.

**CONCLUSION:** The development and progression of esophageal cancer are related to the inactivation of *RIZ1*. Virus infection may also be an important factor.

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**Key words:** Retinoblastoma protein-interacting zinc finger gene 1; Microarray; Nude mice; Esophageal squamous cell carcinoma cells

**Core tip:** Retinoblastoma protein-interacting zinc finger gene 1 (*RIZ1*) transfection induced widespread changes in gene expression profile of cell line TE13, with 960 genes upregulated and 1163 downregulated. Most of the differentially expressed genes are associated with cell development, supervision of viral replication, lymphocyte costimulatory, and immune system development in esophageal cells. *RIZ1* gene may be involved in multiple cancer pathways, such as cytokine receptor interaction and transforming growth factor beta signaling. Virus infection may also be an important factor in

## the development of esophageal cancer.

Dong SW, Li D, Xu C, Sun P, Wang YG, Zhang P. Alteration in gene expression profile and oncogenicity of esophageal squamous cell carcinoma by *RIZ1* upregulation. *World J Gastroenterol* 2013; 19(37): 6170-6177 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i37/6170.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6170>

## INTRODUCTION

Esophageal cancer is one of the most common forms of cancer in the world and is a leading cause of cancer deaths in China and other developing countries. To date, the mechanisms of esophageal cancer are unclear. Tumor occurrence and development are regulated by a variety of oncogenes and tumor suppressor genes<sup>[1-3]</sup>, including the putative tumor suppressor gene, Retinoblastoma protein-interacting zinc finger gene 1 (*RIZ1*). The *RIZ* gene has two expression products: *RIZ1*, which is believed to be a histone methyltransferase and acts on the locus of H3K9; and *RIZ2*, which lacks the PR-domain of *RIZ1*. Abnormal expression of *RIZ1* has been found to be associated with tumor invasion and malignancy<sup>[4-10]</sup>.

Our group has previously reported that *RIZ1* expression level is lower in esophagus carcinoma than in adjacent noncancerous tissues<sup>[11]</sup>, and is related to methylation of CpG islands<sup>[12]</sup>. In addition, by constructing human *RIZ1* eukaryotic expression vectors to transfect human esophageal squamous cell carcinoma (ESCC) cell line TE13, we were able to report that upregulation of *RIZ1* can recover tumor suppression activity and that treatment of cell line TE13 by methyltransferase inhibitor 5-aza-CdR reverses the methylation status of the promoter region<sup>[13]</sup>. In order to investigate *RIZ1*-mediated changes in gene expression of esophageal cancer, we compared the gene expression profile of TE13 cells transfected with *RIZ1* with those of negative control cells. The resulting changes in oncogenicity were analyzed *in vitro* and by animal experimentation.

## MATERIALS AND METHODS

### Ethics

The animal study proposal was approved by the Tianjin Medical University General Hospital Ethics Committee with the permit number: 2012-021. All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

### Cell culture and transfection

Human ESCC cell line TE13 was purchased from ATCC (Rockville, MD, United States) and cultured in RPMI-1640 (HEPES 4.76 g/NaCO<sub>3</sub> 2.0 g/RPMI-1640

10.4 g/ddH<sub>2</sub>O 1000 mL) media supplemented with 10% new-born bovine serum, 2 mmol/L 1 × L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Life Technologies; NY, United States). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. *RIZ1* eukaryotic expression vector pcDNA3.1(+)/*RIZ1* plasmid had been prepared previously and stored at -80 °C. Cultivated TE13 cells were passaged in 12-inch orifice plates. Passaging was repeated every 2-3 d at 1:10 dilution, and cells were lifted by trypsin digestion. When the cells were at the log phase, they were transfected using the classical liposome method by adding 2 µL Lipofectamine 2000 (Life Technologies; NY, United States). Experimental and control groups were transfected with pcDNA3.1(+)/*RIZ1* and pcDNA3.1(+), respectively. The media were changed after 6 h and the cells were washed in phosphate buffered saline (PBS), harvested and counted. After mixing with Trizol Reagent (Life Technologies, NY, United States), the cells were incubated at room temperature for 5 min, then transferred to liquid nitrogen. The resultant cDNA was taken and 0.75 µL was mixed with 2 × SYBR Premix Ex Taq™ (Takara). The following primer sets (10 µmol/L) were used: *RIZ1*, forward 5'-TCTGCTGTTGACAAGACCC-3', reverse 5'-GCATCAATGCACATCCATC-3'; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), forward 5'-ACCCAGAAGACTGTGGATGG-3', reverse 5'-TTCAGCTCAGGGATGACCTT-3'. Amplification was carried out using LightCycler real-time polymerase chain reaction (PCR) system (Roche, United States), according to the manufacturer's protocol. Each sample was run in triplicate for each gene. An initial denaturation step at 95 °C for 5 min was followed by 40 denaturation cycles at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. A solubility temperature curve assay was constructed and the *RIZ1* and *GAPDH* Ct-values for each group were recorded and compared. The *RIZ1* mRNA relative quantitation formula: ( $2^{-\Delta\Delta Ct}$ , × 100%), was applied to evaluate whether transfection was successful. Duplicate detections were performed in triplicate, through  $2^{-\Delta\Delta Ct}$ , to calculate mean ± SD.

### Microarray analysis

Total RNA was extracted using Trizol Reagent (Life Technologies; NY, United States). Quality control was achieved by utilizing the Agilent Bioanalyzer 2100 (Agilent Technologies; United States). Purification was achieved using the RNeasy mini kit and RNase-Free DNase Set (QIAGEN, Germany). Agilent's Low Input Quick Amp Labeling Kit, one-color and full genome chip (4 × 44K, design ID: 014850) were used to amplify and mark the mRNA according to the manufacturer's protocols. The cRNA was purified and conjugated using the RNeasy mini kit. Agilent's Gene Expression Hybridization Kit with a sample quantity of 1.65 µg cRNA was employed for gene chip hybridization for 17 h in a hybridization oven at < 65 °C and 10 rpm, following Agilent's protocol. Slides were washed in staining dishes (Thermo Shandon,

PA, United States) with Gene Expression Wash Buffer Kit. After completion of hybridization, gene chip scanning was performed using an Agilent Microarray Scanner. The software was adjusted to set the Dye channel to Green; scan resolution to 5  $\mu\text{m}$ ; and PMT to 100%, 10%, 16 bit. Data was read by Feature Extraction software v.10.7 and Gene Spring Software v.11.0, and uniformly processed by a Quantile algorithm. Data analysis was carried out using an online analysis system (SAS) (Shanghai Bohao Company, China). Fold changes  $\geq 2$  (upregulated) or  $\leq 0.5$  (downregulated) were set to select differentially expressed genes. Gene ontology (GO) functional analysis, chromosomal assignment, and pathway analysis were then performed. Several representative genes were selected for verification of the gene chip scan results by reverse transcription PCR (RT-PCR).

### Animal experimentation

**Purchase and feeding:** Eighteen BALB/c nude mice (female; aged 4–6 wk; weight, 17–21 g), were purchased from the Experimental Animal Center of the Academy of Military Medical Sciences, Chinese People's Liberation Army, under license No. SCXK-(army)-2007-004. Nude mice were fed in the Tianjin Medical University General Hospital SPF rearing chamber with sterilized standard feed and sterile water. Animal experimentation and protocol were carried out in accordance with medical ethical standards.

**Subcutaneous transplantation:** Cultured cells were adjusted to a final concentration of  $1 \times 10^7$  cells/mL in sterilized PBS; 0.2 mL was injected into the right armpit of each nude mouse and their general condition and tumor growth after inoculation were observed. The large and small tumors in diameter were measured every three days, tumor size was calculated and a tumor growth curve plotted. The antitumor rate was calculated using the following formulae: Volume =  $0.5 \times \text{small diameter} \times (\text{large diameter})^2$ ; antitumor rate =  $(1 - \frac{\text{the experimental group volume}}{\text{the control group volume}}) \times 100\%$ .

**Animal grouping and treatment:** The nude mice were divided into three groups using a random digital method. Five mice from each group, with the maximum and minimum tumor volumes, were separated and the remaining mouse was removed. The blank control group was injected with 100  $\mu\text{L}$  normal saline. The pcDNA3.1(+) group was injected with 20  $\mu\text{L}$  liposome, to which 20  $\mu\text{g}$  empty plasmid, adjusted to a final volume of 100  $\mu\text{L}$  in DMEM, was added. The pcDNA3.1(+)/RIZ1 recombinant plasmid group was injected with 20  $\mu\text{L}$  liposome to which 20  $\mu\text{g}$  RIZ1 recombinant plasmid, adjusted to a final volume of 100  $\mu\text{L}$  in DMEM, was added. Each group was injected every two days using the multi-point injection method, and the treatment was repeated 5 times. Seven days after the final injection, the mice were killed by cervical dislocation and the tumor tissue was removed, weighed and used for subsequent experimentation.

### RT-PCR

RT-PCR was carried out as described above, and 12 mL of the reaction products were analyzed by electrophoresis on a 20 g/L agarose gel. The electrophoresis images were scanned by UV spectrophotometry (Beckman Coulter Inc., Brea, CA, United States).

### Western blotting

Tissue from each group was homogenized in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; 1 mmol/L EDTA; 1 mmol/L PMSF; 1 mg/mL Aprotinin). The supernatant was collected and protein concentrations were determined by a bicinchoninic acid (BCA) protein assay kit (Pierce; IL, United States); 30  $\mu\text{g}$  of whole-cell lysate was separated on 8% SDS-PAGE gels, transferred to nitrocellulose (NC) membranes (Amersham Biosciences; New Jersey, United States), and immunoblotted with the following antibodies: anti- $\beta$ -actin (ABCAM; United Kingdom), control; primary antibodies, 1:2000 dilution (ABCAM; United Kingdom); secondary antibodies Goat Anti-Mouse, 1:5000 dilution (ABCAM; United Kingdom). The films were analyzed by a PowerLook scanner (UMAX) and quantified by Image Quant software (GE; United States). The control experiments (TE13 cells; TE-13 cells transfected with blank plasmid) were treated by the same method. Relative expression of RIZ1 = gray value of RIZ1 protein/gray value of  $\beta$ -actin.

## RESULTS

### Transfection

The melting curve peaks for RIZ1 and GAPDH transcript products were at 80  $^{\circ}\text{C}$  and 82.5  $^{\circ}\text{C}$ , respectively (Figure 1), giving Ct-values for the two groups. The relative expression levels were calculated using the real-time PCR relative quantitative formula. RIZ1 gene expression levels were compared with SPSS v.13.0 statistical software. The results showed that the mRNA expression level in the experimental group was higher than in the control groups (Figure 2), indicating that transfection had been successful ( $P \leq 0.01$ ).

### Alteration of gene expression profile

Table 1 gives the 2100 results for  $RIN \geq 7.0$  and  $28S/18S \geq 0.7$ , therefore qualifying the samples without degradation. The initial scanned single fluorescence chip data (Figure 3A) were standardized and converted to logarithmic values. A scatter plot was constructed with a two-dimensional rectangular coordinate plane (Figure 3B).

### GO and microarray analysis

The SAS system was used for GO analysis of the differentially expressed genes and  $P \leq 0.05$  was considered to be statistically significant (Table 2). The microarray data showed that 2123 genes were differentially expressed in the pcDNA3.1(+)/RIZ1 transfected cells with fold

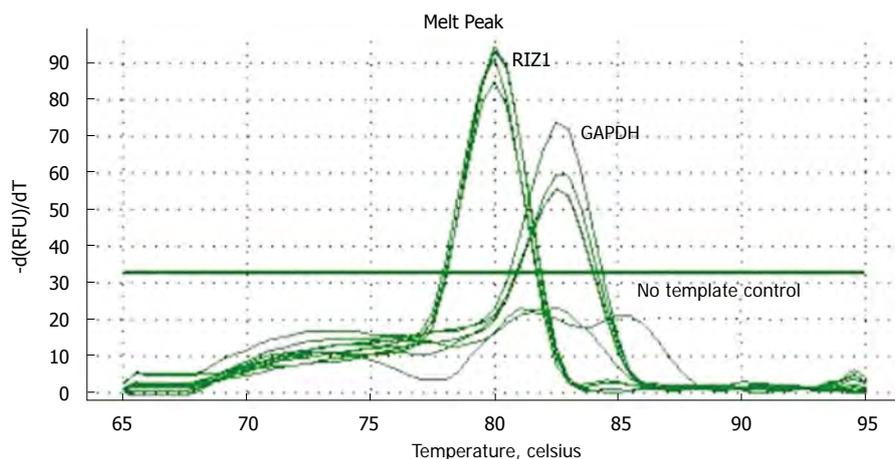


Figure 1 Solubility temperature curves for RIZ1 and glyceraldehyde 3-phosphate dehydrogenase showing that transfection was successful. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

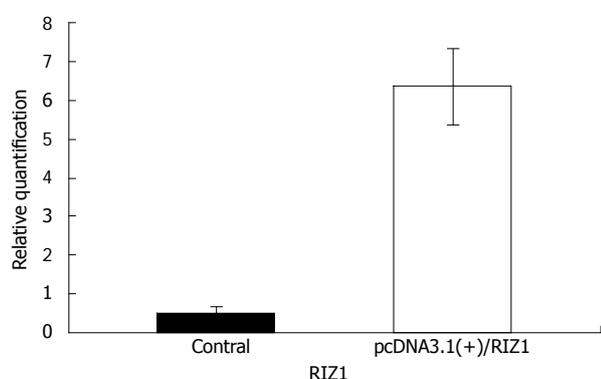


Figure 2 Histogram of RIZ1 gene mRNA expression levels in the blank control group and pcDNA3.1(+)/RIZ1 recombinant plasmid group, clearly showing that gene expression is higher in the pcDNA3.1(+)/RIZ1 group ( $P \leq 0.01$ ).

Table 1 Sample qualification					
Sample number	Concentration ( $\mu\text{g}/\mu\text{L}$ )	$A_{260}/A_{280}$	2100 result		Results
			RIN	28S/18S	
1	0.816	1.94	8.9	1.5	Qualified
2	0.865	1.93	8.9	1.5	Qualified

changes  $> 2$  ( $P < 0.05$ ) compared to control samples. Of these, 960 genes were upregulated, of which 654 were known genes (1.70%, 654/38500) and 306 were unknown; 1163 genes were downregulated, of which 719 were known genes (1.87%, 719/38500) and 444 were unknown. Subsequent analyses were primarily carried out on annotated genes. The gene chip results were confirmed by RT-PCR (Figure 4).

### Pathway analysis

Many of the identified genes are associated with cell development, virus replication supervision, costimulatory molecule, and immune system development. Further analysis indicated that the RIZ1 gene may participate in multiple signaling pathways ( $P < 0.01$ ), some of which

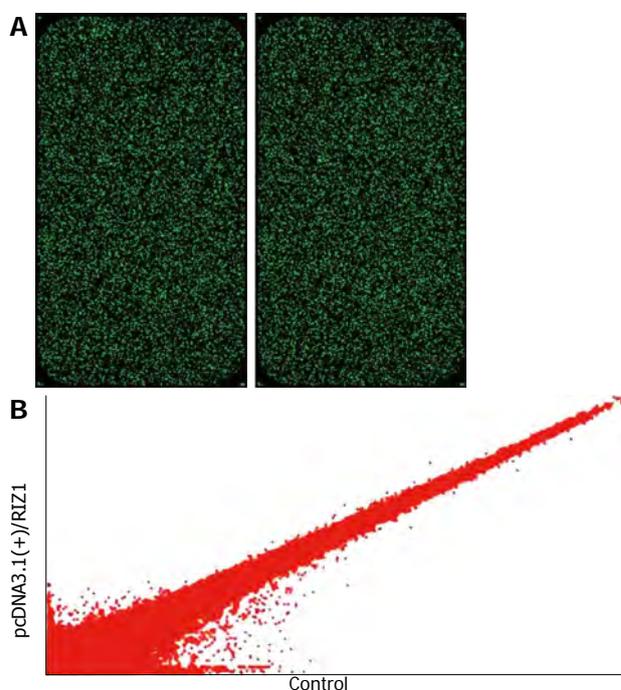


Figure 3 Alteration of gene expression profile. A: Single fluorescence chip images for microarray analysis; B: The data were used to construct the scatter plot. Hybridization signal strength scatter diagram. Each point in the scatter diagram represents a probe point on the chip. The position of each point is identified by an  $x$  and  $y$  coordinate: the  $x$ -coordinate gives the standardized signal value on the control chip; the  $y$ -coordinate gives the standardized signal value on the sample chip. The scatter plot is used to assess the centralized tendency of two sets of data.

are given in Table 3.

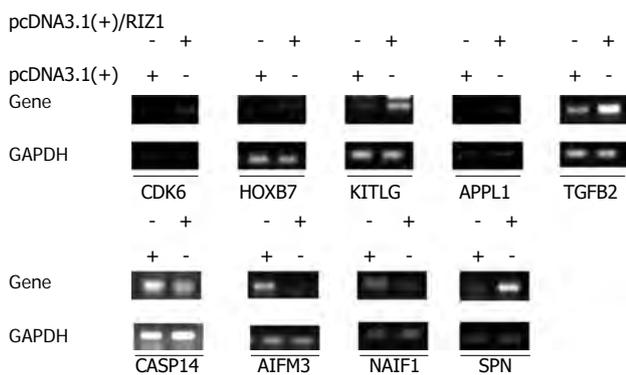
### Transplantation and tumor growth

Transplantation of human esophageal cancer TE13 cells into nude mice was successful as shown in Figure 5. Tumor volumes were compared among the three groups (Figure 6A). The tumor growth rate curves revealed that tumor growth was slower in the pcDNA3.1(+)/RIZ1 group, with a shallower growth rate curve, than in the

**Table 2** Gene ontology analysis of differentially expressed genes

GO ID	Name	Hits	Total	Percentage	P value
GO: 0048468	Cell development	81	832	9.74%	0.0288
GO: 0050792	Regulation of viral reproduction	5	20	25.00%	0.0304
GO: 0031294	Lymphocyte costimulation	3	8	37.50%	0.0397
GO: 0002520	Immune system development	38	358	10.61%	0.0428

Hits: Number of differentially expressed genes in the pathway; Total: The total number of genes in the pathway; Percentage: Percentage hits in the pathway (Hits/Total); P: Enrichment P value; GO: Gene ontology.



**Figure 4** Comparison between results from the gene chip and the reverse transcription-polymerase chain reaction. Out of the nine genes tested, eight yielded consistent results for both reverse transcription-polymerase chain reaction and gene chip. The five upregulated genes are CDK6, HOXB7, KITLG, APPL1, TGFB2; and the three downregulated genes are CASP14, AIFM3 and NAIF1; however, in the case of service principal name (SPN), the reverse transcription-polymerase chain reaction results indicate upregulation, whereas the gene chip results indicate downregulation.

other two groups (Figure 6B). The following tumor volumes were recorded after 26 d:  $1.025 \pm 0.018 \text{ cm}^3$  for the pcDNA3.1(+)/RIZ1 group,  $2.208 \pm 0.092 \text{ cm}^3$  for the blank control group, and  $1.980 \pm 0.089 \text{ cm}^3$  for the pcDNA3.1(+) group, showing that the tumor volume of the pcDNA3.1(+)/RIZ1 group was significantly lower than that of the other two groups ( $P < 0.05$ ). In contrast, there was no significant difference between the blank control and pcDNA3.1(+) groups ( $P > 0.05$ ).

**Electrophoresis**

RNA was extracted from the transplanted tumors for RT-PCR, and the products were analyzed by electrophoresis: GAPDH was identified at 125 base pairs (bp) and RIZ1 at 167 bp. The intensity of the GAPDH band was similar among the three groups; however, the intensity of the RIZ1 band was greatest in the pcDNA3.1(+)/RIZ1 group, showing that RIZ1 expression level was higher than in the other two groups (Figure 7).

**Western blotting**

The housekeeping gene,  $\beta$ -actin, was used as a control for Western blot analysis (Figure 8). The results showed no

**Table 3** RIZ1 pathway analysis (selected pathways)

Name	Hits	Total	Percentage	P value	q
Cytokine-cytokine receptor interaction	25	276	9.06%	0	0
TGF-beta signaling pathway	10	85	11.76%	3.00E-04	5.00E-04
MAPK signaling pathway	20	271	7.38%	2.00E-04	5.00E-04
Pathways in cancer	20	328	6.10%	0.0018	0.0015

Hits: Number differentially expressed genes in the pathway; Total: Total number of genes in the pathway; Percentage: Percentage of hits in the pathway (Hits/Total); P: Enrichment P value; q: False discovery rate; TGF: Transforming growth factor; MAPK: Mitogen-activated protein kinase.



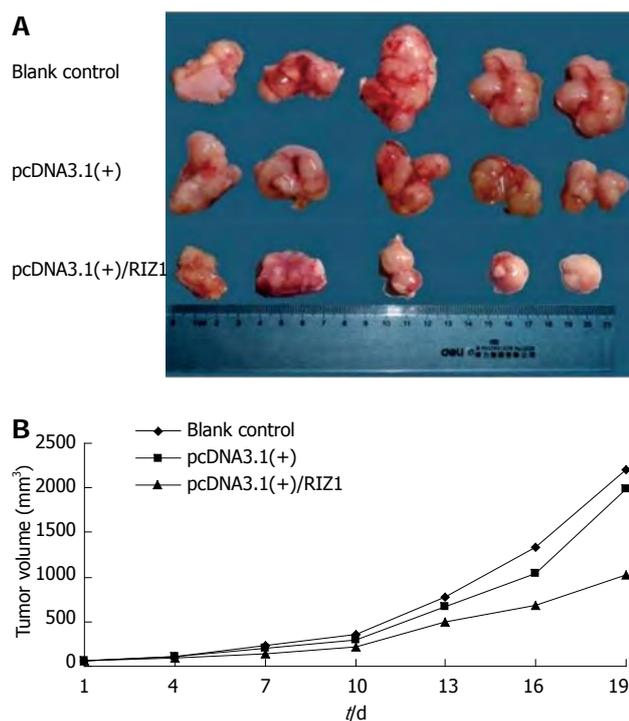
**Figure 5** Photograph showing tumor development in a nude mouse, confirming successful transplantation of human esophageal cancer into an animal model.

obvious difference in density of the  $\beta$ -actin bands among the three groups. In contrast, the density of the RIZ1 band was higher in the pcDNA3.1(+)/RIZ1 group than in the blank control and pcDNA3.1(+) groups, indicating that RIZ1 had been successfully expressed in the tumor tissues.

**DISCUSSION**

Esophageal cancer is the world's most common cancer of the digestive system, with 70% of incidences occurring in China. China also has the highest incidence and mortality rates for both men and women. Recent statistics on morbidity and mortality for cancer patients show that esophageal cancer is the 6<sup>th</sup> most common form of cancer, and the 4<sup>th</sup> highest cause of cancer death-related in China<sup>[14-18]</sup>. Furthermore, the incidence rate is higher in rural areas than in urban areas. Esophageal cancer can be divided into two pathological types: ESCC and esophageal adenoid carcinoma. In China, ESCC accounts for 90% of esophageal cancers, in contrast to Western countries.

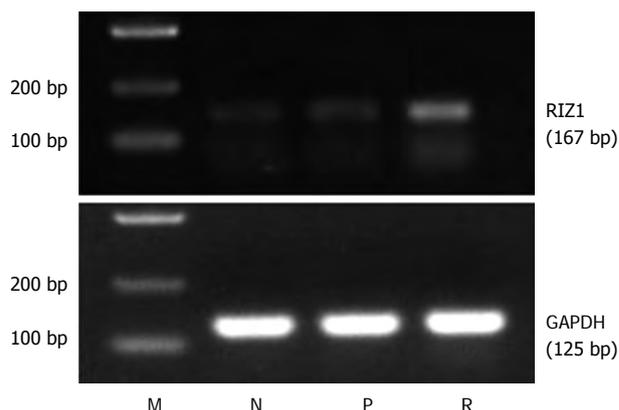
Treatment of esophageal carcinoma is a long-term project; however, by combining several different treatment types, the quality of life of the patients can be greatly improved. The purpose of our study is to enhance the understanding of ESCC development and mechanisms at the genetic level in order to advance clinical therapies. RIZ1 is one of the most effective tumor sup-



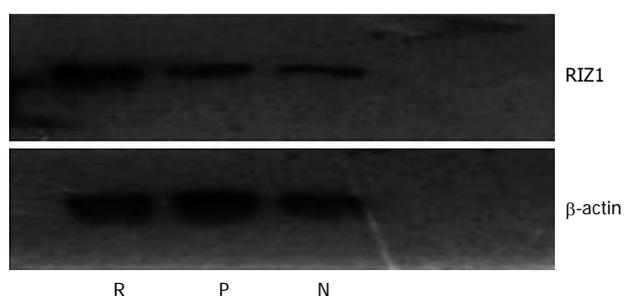
**Figure 6** Transplantation and tumor growth. A: Photograph comparing esophageal squamous cell carcinomas removed from nude mice of the three groups after 26 d; B: Growth rate curves showing the increase in tumor volumes with time. The shallower curve in the pcDNA3.1(+)/RIZ1 group, compared to the blank control and pcDNA3.1(+) groups, indicates slower growth.

pression genes, therefore failure of RIZ1 expression can lead to the development of many forms of cancer<sup>[19-25]</sup>. Our group has carried out a number of studies on the *RIZ1* gene<sup>[11,12]</sup>; however, further research is required. One key research area is the introduction of a foreign gene into the tumor tissue, which is then able to express stably; eukaryotic expression vector is an ideal choice. The liposome mediated method was adopted because it has the advantages of high transfection efficiency, low immunogenicity, simple manipulation, and can be applied to a wide variety of cells.

We constructed the *RIZ1* gene eukaryotic expression vector pcDNA3.1(+)/RIZ1 using an established molecular biology technique; empty plasmid, pcDNA3.1(+), was used as a negative control. After transfection into ESCC cell line TE13, *in vivo* experiments and gene chip analyses were carried out, as described in Materials and Methods. Our results showed that the xenograft in nude mice had a slower growth rate, and lower tumor volume and mass, in the pcDNA3.1(+)/RIZ1 group than in the blank control and pcDNA3.1(+) groups. This suggests that RIZ1 has a restraining effect on ESCC tumor growth. In contrast, the growth curves for the blank control and pcDNA3.1(+) groups were approximately parallel, indicating that these groups had no restraining effect. Secondly, after transfection, the gene expression profile of the cell line TE13 underwent extensive changes, with a total of 2123 differentially expressed genes, including 1163 downregulated and 960 upregulated. We found that



**Figure 7** Electrophoresis gel image showing bands for glyceraldehyde 3-phosphate dehydrogenase (125 bp) and RIZ1 (167 bp). The intensity of the RIZ1 band is greatest in the pcDNA3.1(+)/RIZ1 group. M: Marker (D2000); N: Blank control; P: pcDNA3.1(+); R: pcDNA3.1(+)/RIZ1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.



**Figure 8** Western blot image for RIZ1 protein expression with  $\beta$ -actin as the control. The density of the RIZ1 band in the pcDNA3.1(+)/RIZ1 group is higher than in the other groups, indicating increased expression. R: pcDNA3.1(+)/RIZ1; N: Blank control; P: pcDNA3.1(+).

many of these genes are involved in cell development, lymphocyte costimulatory, immune system development, and interestingly, in the supervision of viral replication. This is consistent with the results from Dąbrowski *et al.*<sup>[13]</sup> who reported that low-risk type human papilloma virus may be one of the auxiliary activated or carcinogenic factors in ESCC occurrence and development. Vaiphei *et al.*<sup>[26]</sup> also found that the infection rates of human papilloma virus in ESCC patients was as high as 87%, especially in patients with two or more types of phenotypic mixed infection. Persson *et al.*<sup>[27]</sup> reported that HIV infection increases the risk of esophageal cancer. All these reports indicate that the occurrence and development of esophageal cancer may be related to virus infection; however, the pathophysiological mechanisms are poorly understood and require further research.

In conclusion, we propose that the occurrence of esophageal cancer is a consequence of widespread alterations in gene expression, involving multiple functions and signaling pathways with roles in tumor development, some of which are synergistic or antagonistic. We also speculate that the most probable signaling pathways in ESCC affected by these genes are the cytokine receptor interaction and transforming growth factor pathways.

Therefore, we recommend that future research should be directed towards better understanding of the relationship between the *RIZ1* gene and ESCC and the mechanism and role of virus infection in ESCC occurrence and development.

## COMMENTS

### Background

Esophageal cancer is one of the most common forms of cancer in the world and is a leading cause of cancer deaths in many developing countries. China is a country with a high incidence of esophageal cancer, and the pathological type is mainly the squamous cell carcinoma, which is different from the Western countries where adenocarcinoma is reported to be the main pathological type. To date, the mechanisms of esophageal cancer are unclear. Tumor occurrence and development are regulated by a variety of oncogenes and tumor suppressor genes, including the putative tumor suppressor gene, retinoblastoma protein-interacting zinc finger gene 1 (*RIZ1*).

### Research frontiers

*RIZ1* expression is lower in esophagus carcinoma and is related to methylation of CpG islands. In addition, by constructing human *RIZ1* eukaryotic expression vectors to transfect human esophageal squamous cell carcinoma (ESCC) cell line TE13, the authors were able to report that upregulation of *RIZ1* can recover tumor suppression activity and that treatment of cell line TE13 by methyltransferase inhibitor 5-aza-CdR reverses the methylation status of the promoter region.

### Innovations and breakthroughs

In order to investigate *RIZ1*-mediated changes in gene expression of esophageal cancer, the authors compared the gene expression profile of TE13 cells transfected with *RIZ1* with that of negative control cells. The resulting changes in oncogenicity were analyzed *in vitro* and by animal experimentation. They found that the development and progression of esophageal cancer are related with the inactivation of *RIZ1*. In addition, virus infection may also be an important factor.

### Applications

*RIZ1* is one of the most effective tumor suppression genes, therefore failure of *RIZ1* expression can lead to the development of ESCC, which is expected to be a molecular biological parameter for early diagnosis.

### Peer review

This paper reports that the development and progression of esophageal cancer is relevant to the inactivation of *RIZ1*. In addition, virus infection may also be an important factor.

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## Cytokeratin 8 is increased in hepatitis C virus cells and its ectopic expression induces apoptosis of SMMC7721 cells

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### Abstract

**AIM:** To investigate cytokeratin 8 (*CK8*) overexpression during hepatitis C virus (HCV) infection and its pathogenesis, and the effect of ectopic *CK8* expression on hepatoma cell lines.

**METHODS:** We successfully established an *in vitro* HCV cell culture system (HCVcc) to investigate the different expression profiles of *CK8* in Huh-7-HCV and Huh-7.5-HCV cells. The expression of *CK8* at the mRNA level was determined by real-time polymerase chain reaction (RT-

PCR). The expression of *CK8* at the protein level was evaluated by Western blotting. We then constructed a eukaryotic expression combination vector containing the coding sequence of human full length *CK8* gene. *CK8* cDNA was amplified by reverse transcription-PCR and inserted into pEGFP-C1 and the positive clone pEGFP-*CK8* was obtained. After confirming the sequence, the recombinant plasmid was transfected into SMMC7721 cells with lipofectamine2000 and *CK8* expression was detected using inverted fluorescence microscopy, RT-PCR and Western blotting. Besides, we identified biological function of *CK8* on SMMC7721 cells, including cell proliferation, cell cycle and apoptosis detection.

**RESULTS:** RT-PCR showed that the expression level of *CK8* in Huh-7-HCV and Huh-7.5-HCV cells was 2.88 and 2.95 times higher than in control cells. Western blot showed that *CK8* expression in Huh-7-HCV and Huh-7.5-HCV cells was 2.53 and 3.26 times higher than that in control cells, respectively. We found that *CK8* at mRNA and protein levels were both significantly increased in HCVcc. *CK8* was up-regulated in SMMC7721 cells. *CK8* expression at the mRNA level was significantly upregulated in SMMC7721/pEGFP-*CK8* cells. *CK8* expression in SMMC7721/pEGFP-*CK8* cells was 2.69 times higher than in SMMC7721 cells, and was 2.64 times higher than in SMMC7721/pEGFP-C1 cells. *CK8* expression at the protein level in SMMC7721/pEGFP-*CK8* cells was 2.46 times higher than in SMMC7721 cells, and was 2.29 times higher than in SMMC7721/pEGFP-C1 cells. Further analysis demonstrated that forced expression of *CK8* slowed cell growth and induced apoptosis of SMMC7721 cells.

**CONCLUSION:** *CK8* up-regulation might have a functional role in HCV infection and pathogenesis, and could be a promising target for the treatment of HCV infection.

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**Key words:** Hepatitis C virus cell culture system; Cytoker-

atin 8; Up-regulation; Eukaryotic expression; Apoptosis

**Core tip:** In this study, we observed that cytokeratin 8 (CK8) levels are elevated in hepatitis C virus (HCV) cell culture system and its ectopic expression decreased the proliferation and induced apoptosis of SMMC7721 cells. CK8 up-regulation might have a functional role in HCV infection and pathogenesis, and could be a promising target for the treatment of HCV infection.

Sun MZ, Dang SS, Wang WJ, Jia XL, Zhai S, Zhang X, Li M, Li YP, Xun M. Cytokeratin 8 is increased in hepatitis C virus cells and its ectopic expression induces apoptosis of SMMC7721 cells. *World J Gastroenterol* 2013; 19(37): 6178-6187 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6178.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6178>

## INTRODUCTION

Hepatitis C virus (HCV) infection is a significant global healthcare burden<sup>[1]</sup>. Current estimation suggests that a minimum of 3% of the world's population is chronically infected, with a prevalence of up to 170 million people<sup>[2,3]</sup>. However, the mechanism of HCV infection is not fully understood. Recently, the development of HCV replicon technology has accelerated the understanding of the mechanism underlying HCV infection<sup>[4,5]</sup>. It has been reported that there were more than 100 abnormal expression proteins in HCV infected cells and hepatitis C patients<sup>[6-10]</sup>. Studies determining the changes in protein expression associated with HCV infection will help elucidate host/virus interactions, and provide further insight to HCV pathogenesis.

Cytokeratin 8 (CK8) is the major component of the intermediate filament cytoskeleton, belonging to the type-II keratin, and is primarily expressed in the epithelia of liver, intestine, and exocrine pancreas<sup>[11,12]</sup>. CK8 plays a crucial role in maintaining the structural integrity and the mechanical properties of cells<sup>[13]</sup>. Recent studies have suggested that CK8 is involved in several liver diseases. CK8 knock-out mice develop liver hemorrhage and are more susceptible to liver injury<sup>[14,15]</sup>. Some variants of CK8 are associated with disease severity and progression in patients with chronic liver diseases<sup>[16,17]</sup>. Thus, we hypothesized that CK8 contributed to cellular pathological processes and the infection and pathogenesis of HCV, leading to liver injury and chronic liver diseases.

In this study, we established an *in vitro* HCV cell culture system (HCVcc) and investigated whether HCV affects CK8 levels. Simultaneously, we established eukaryotic expression recombination vector containing the full length coding sequence of CK8, then transfected into hepatoma cells *in vitro* and investigated the biological and functional role of CK8 in hepatoma cells.

## MATERIALS AND METHODS

### Construction and identification of HCVcc

Huh-7 and Huh-7.5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids and 1 × penicillin-streptomycin-glutamine. Plasmid pFL-J6/JFH, containing a chimeric full length HCV genome, was kindly provided by Professor Charles M Rice from Rockefeller University. Plasmid pFL-J6/JFH, containing a single Xba I restriction site and T7 RNA polymerase start site, is the chimera of HCV J6 strain (5'-NCR-NS2) and JFH strain (NS3-3'-NCR). Subsequently, plasmid pFL-J6/JFH encoding the full length HCV chimeric genome was transcribed to HCV RNA *in vitro*. HCVcc was established by electroporation of HCV RNA into Huh-7 and Huh-7.5 cells.

Huh7 and Huh-7.5 were used as negative controls of HCVcc. Huh-7-HCV and Huh-7.5-HCV cells were maintained under the same condition as Huh-7 and Huh-7.5 cells. Cells were cultured in an incubator at 37 °C supplemented with 5% CO<sub>2</sub>. During the cell culture, the supernatant of cell culture was collected at 24, 72 and 96 h after electroporation in order to determine the HCV copies. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine HCV copy number. At approximately 72 h after transfection, cells were washed three times with 1 × phosphate-buffered saline (PBS) and then harvested. In addition, indirect immunofluorescence was used to observe the expression of HCV core protein. Mouse monoclonal HCV core protein antibody (Novus Biologicals, United States) was used as the primary antibody, and goat anti-mouse conjugated with Fluorescein Isothiocyanate (FITC) was used as the secondary antibody. The harvested cells were fixed with 3% glutaraldehyde at 4 °C for 24 h, then washed twice by 0.1 mol/L arsenic acid dimethyl sodium buffer (pH 7.4) at 4 °C, fixed by 1% osmium tetroxide for 1 h, gradient acetone dehydration, embedded by Epon812, sliced by ultra-thin LKB-V slicer. H-7650 transmission electron microscope (HITACHI, Japan) was also used to observe the morphology of the viral particles and intracellular ultrastructure changes.

### Total RNA isolation, cDNA synthesis and RT-PCR

Total RNA was extracted from cells by TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol. A two-step reverse transcription PCR was performed. The first-strand cDNA was synthesized from 1 µg of total RNA with AMV Reverse Transcriptase<sup>b</sup> (TAKARA, Japan). To investigate the expression of CK8 at the mRNA level, the expression of CK8 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) genes was quantified by RT-PCR, and GAPDH was used as an internal control. A total of 20 ng cDNA was used as template in the reaction. All RT-PCR assays were performed

**Table 1 Primers used for real-time polymerase chain reaction and high fidelity**

Name	Forward primer (5'-3')	Reverse primer (5'-3')
CK8 (172 bp)	AGCTGGAGICTCGCTGGAA	TGTGCCTTGACCTCAGCAATG
GAPDH (138 bp)	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
CK8 (1465 bp)	ATGTCGACATGTCCATCAGGGTGAC	TAGGATCCCTTGGCAGGACGTC

CK8: Cytokeratin 8; PCR: Polymerase chain reaction; GAPDH: Glyceraldehydes-3-phosphate dehydrogenase.

in triplicate using SYBR green incorporation method with Bio-Rad IQ5 Multicolor RT-PCR Detection System (Bio-Rad, United States) based on the manufacturer's protocol. Table 1 shows the sequences of the primer sets for CK8 and GAPDH. Briefly, following a denaturation at 95 °C for 5 s, RT-PCR was carried out with 50 cycles at a melting temperature of 95 °C for 30 s, an annealing temperature of 65 °C for 30 s, and an extension temperature of 72 °C for 10 s. Data analysis was performed using the Sequence Detector System software. The relative quantification was calculated by the  $2^{-\Delta\Delta Ct}$  method with GAPDH as the housekeeping gene and the control cells as the baseline, and the results were expressed as fold-change.

#### Protein extraction, SDS-PAGE and Western blotting

Total proteins were prepared by RIPA cell lysate. Proteins of interest were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% polyacrylamide gel, and 1 mg/mL protein was loaded onto a SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes and then detected by Western blotting under the recommended conditions. Mouse anti-human CK8 IgG (Abcam, United States) was used as the primary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was used as the secondary antibody, and GAPDH was used as the control. The antigen-antibody complex was detected by an enhanced chemiluminescence (ECL) kit following the manufacturer's protocol. The experiments were repeated in triplicate. The chemiluminescent signal of each band was analyzed by gel image analysis system (Syngene, United States).

#### Construction of pEGFP-CK8 recombination vector

The *Bam*H I and *Sal* I restriction sites were introduced into the CK8 coding sequence (CDS) by high fidelity PCR (Thermo, United States). Sequences for the primers are listed in Table 1, with the amplified product being 1465 bp. The CK8 CDS was purified by gel extraction. CK8 CDS and pEGFP-C1 vector (TAKARA, Japan) were digested respectively by the restriction enzyme *Sal* I and *Bam*H I (TAKARA, Japan). The digestion products were examined on 1% agarose gel by electrophoresis. The ligation reaction (Ligation Kit, TAKARA, Japan) was carried out between both of the DNA fragments, followed by transformation into competent *Escherichia coli* DH5 $\alpha$  cells at 37 °C overnight (12-16 h). Colony selection was performed by PCR, and the amplicons were examined on 1% agarose gel by electrophoresis. Plasmid extraction (E.Z.N.A.<sup>®</sup> Endo-Free Plasmid Midi Kit, Omega, United

States) was carried out for positive colonies, and then sequenced and matched by Blast method.

#### Transfection of pEGFP-CK8 vector into SMMC 7721 cells

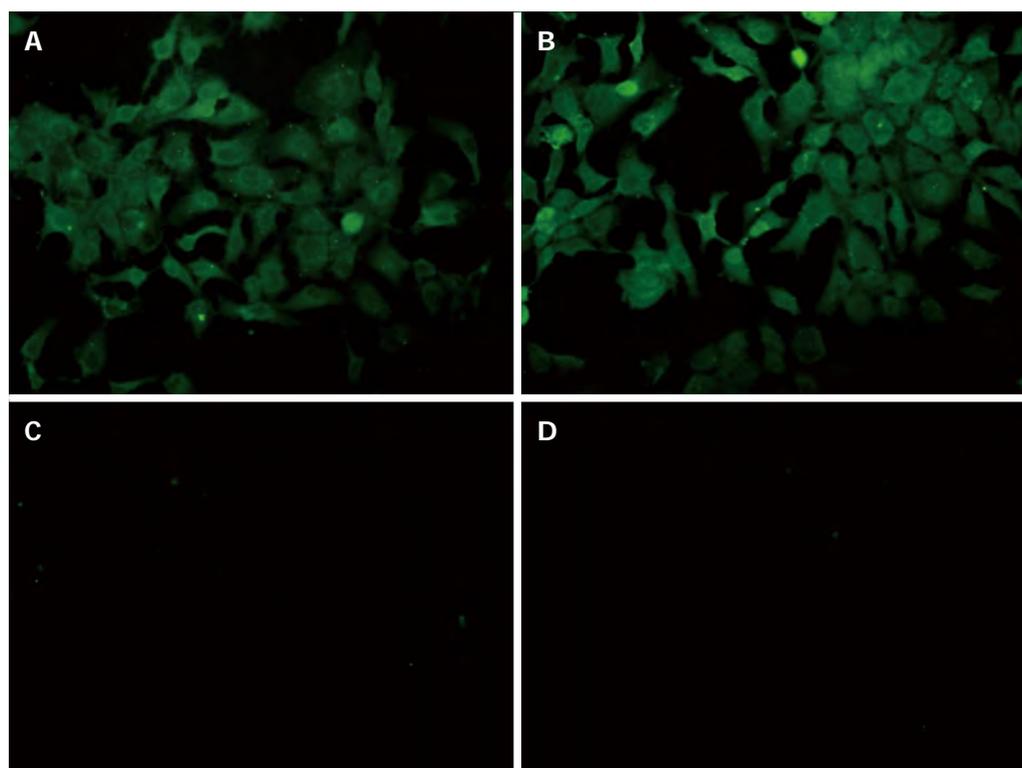
SMMC7721 cells were seeded in 6-well plates in 4 mL of growth medium for 24 h prior to transfection. In each well,  $0.8 \times 10^5$ - $4.0 \times 10^5$  adherent cells were seeded. Four microgram (4.0  $\mu$ g) of DNA (pEGFP-CK8 vector or pEGFP-C1 vector) was diluted in 250  $\mu$ L of serum-free DMEM. Lipofectamine2000 (Millipore, United States) was added (10  $\mu$ L) to the diluted DNA and mixed immediately by pipetting. The mixture was incubated for 25 min at room temperature. The lipofectamine2000/DNA mixture (500  $\mu$ L) was added dropwise to the four wells containing the pEGFP-CK8 plasmid, and another two wells to control cells containing the pEGFP-C1 plasmid. The plate was then gently rocked to achieve even distribution of the complexes and incubated at 37 °C in a 5% CO<sub>2</sub> incubator.

#### Detection assay

The expression and distribution of CK8 was observed under an inverted fluorescence microscope (Nikon eclipse Ti, Japan) 24 h after transfection. Forty-eight hours after transfection, cellular RNA and total cellular proteins were determined by RT-PCR and Western blotting, respectively. Total RNA was extracted from SMMC7721, SMMC7721/ pEGFP-C1, and SMMC7721/pEGFP-CK8 cells by TRIzol reagent. Total proteins were prepared by RIPA cell lysate. Real time PCR assays (SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II, TAKARA, Japan) were performed in triplicate with Bio-Rad iQ5 Multicolor RT-PCR Detection System according to the manufacturer's protocol. Rabbit anti-human IgG (Santa, United States) was used as the primary antibody, goat anti-rabbit IgG conjugated with HRP was used as the secondary antibody and  $\beta$ -actin (Abcam, United States) was used as control. Cells were collected after 24, 48 and 72 h transfection to perform a proliferation assay by MTT reaction (MTT cell proliferation Assay kit, Trevigen, United States). Cells were also collected 48 h after transfection to detect apoptosis (Annexin V-FITC Apoptosis Detection Kit, Abcam, United States) using Flow Cytometry (guava easyCyte HT, Millipore, United States).

#### Statistical analysis

All experiments were performed in triplicate. Representative graphical data are presented as mean  $\pm$  SD. Statistical analyses were performed using the SPSS 10.0 software



**Figure 1** Indirect immunofluorescence detection of hepatitis C virus core proteins (400 ×). A: Huh-7-HCV cells appeared bright green fluorescent, they are HCV core proteins which were labeled with GFP; B: Huh-7.5-HCV cells appeared bright green fluorescent, they are HCV core proteins which were labeled with GFP; C and D: In Huh-7 and Huh-7.5 control cells, there were no green fluorescent, HCV core proteins were not expressed in them.

**Table 2** Detection of hepatitis C virus at RNA level in transfected cellular supernatant

	HCV RNA in supernatant of Huh-7-HCV cells	HCV RNA in supernatant of Huh-7.5-HCV cells
24 h	$5.73 \times 10^5$	$9.48 \times 10^5$
48 h	$1.38 \times 10^6$	$6.40 \times 10^6$
72 h	$3.00 \times 10^4$	$9.29 \times 10^4$
96 h	$6.62 \times 10^5$	$1.43 \times 10^4$

HCV: Hepatitis C virus.

package (SPSS Inc.). We used Student's *t* test. *P* values below 0.05 were considered to be significant.

## RESULTS

### Detection of HCV RNA copies, HCV core protein, and HCV particles

We determined HCV RNA copy number by performing qRT-PCR of viral supernatants obtained from HCV-transfected cells. High-level viral copies in the supernatant of transfected cells were observed at different time-points and reached its peak value at 48 h after transfection (Table 2). Indirect immunofluorescence also showed high expression of HCV core protein in the HCV-transfected cells. Huh-7-HCV and Huh-7.5 HCV cells were also labeled with GFP, further indicating that HCV core protein has been expressed in these cells compared to control cells (Figure 1). Transmission electron microscopy (TEM)

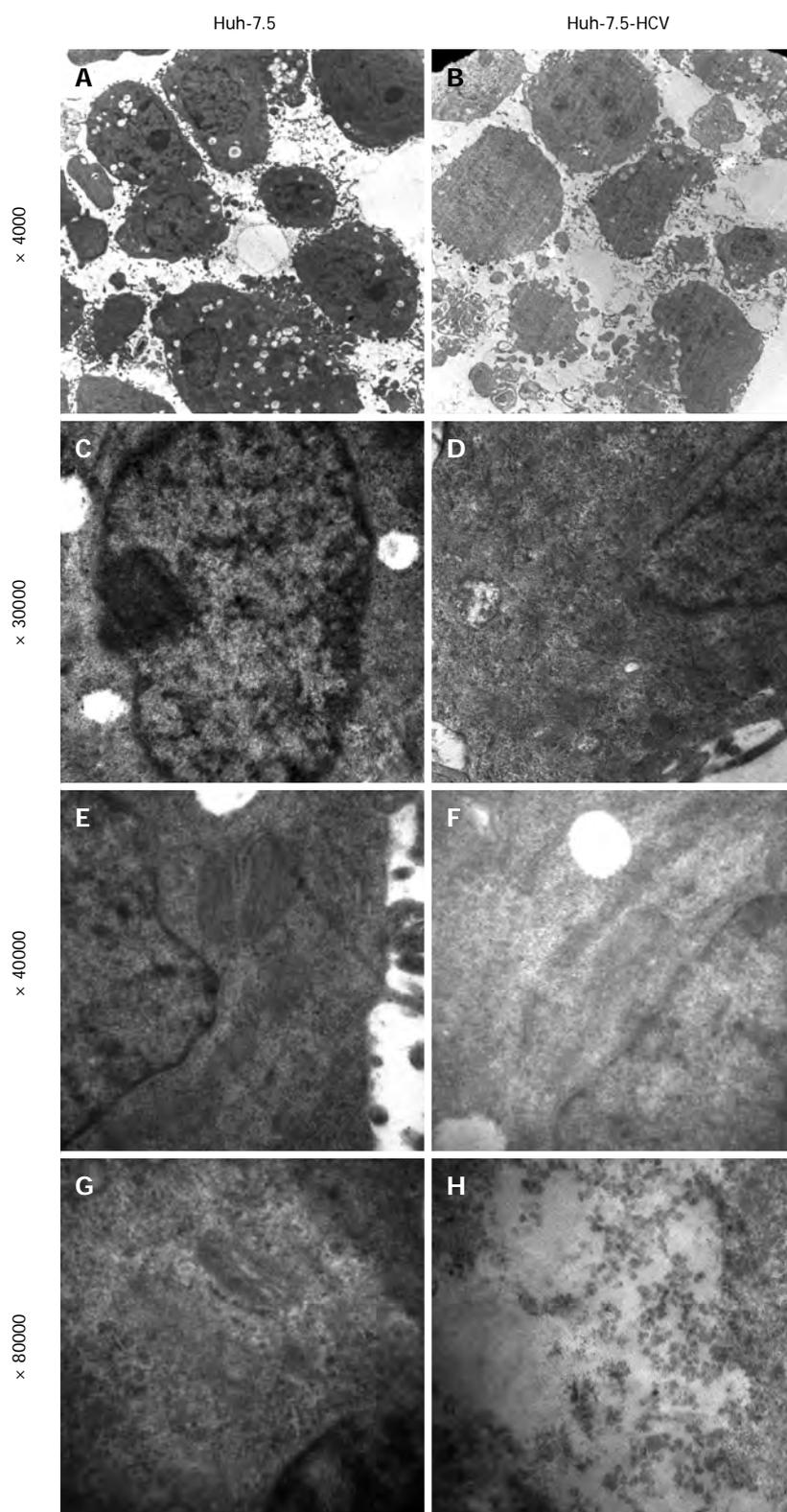
revealed a large number of enveloped or unenveloped virus-like particles (VLPs) in HCVcc. Some characteristic structures of *Flaviviridae* virus infection were observed, including an increased number of endoplasmic reticulum, mitochondrial swelling, cristae disappearance, and cytoplasmic vacuolar structures. Also, a large number of HCV nucleocapsid-like particles of inclusion body were presented in HCVcc cells (Figure 2). Viral-like particles were not seen in the control cells. Moreover, hyperplasia, vacuolar membrane structure, and formation of inclusion bodies were not observed in the control cells.

### Increased CK8 levels in HCVcc cells by RT-PCR

Extracted total cellular RNA was examined by electrophoresis on a 0.8% non-denaturing agarose gel. A 172 bp fragment of *CK8* was successfully amplified by PCR without unspecific amplification. The melting and amplification curves of *CK8* expression indicated that the primers were properly designed. *CK8* expression in Huh-7-HCV cells was 2.88 times higher than that in Huh-7 cells, and *CK8* expression in Huh-7.5-HCV cells was 2.95 times higher than that in Huh-7.5 cells (Figure 3). Therefore, *CK8* was significantly highly expressed in HCVcc cells.

### Increased CK8 levels determined by Western blotting of HCVcc cells

By Western blotting, we showed that the ratio of *CK8*/GAPDH was  $0.079 \pm 0.004$  and  $0.031 \pm 0.003$  in Huh-7-HCV cells and Huh-7 cells, respectively, which was 2.53



**Figure 2** Transmission electron microscopy of hepatitis C virus-transfected Huh7.5 cells ( $\times 4000$ ,  $\times 30000$ ,  $\times 40000$ ,  $\times 80000$ ). A, C, E, G: Control human hepatoma cells, no virus-like particles, mitochondrial and endoplasmic reticulum are normal; B, D, F, H: HCV-transfected human hepatoma cells, human hepatoma cells have large deformed nuclei, and cultured cells prone to exist large vacuoles; D shows mitochondrial swelling and cristae disappearance; F shows the rough endoplasmic reticulum increased; H shows spherical structures of electron density, diameter is between 30-50 nm.

times higher. Furthermore, the ratio of *CK8*/*GAPDH* was  $0.105 \pm 0.004$  in Huh-7.5-HCV cells, which was significantly higher than in Huh-7.5 cells ( $0.032 \pm 0.002$ )

and expression was 3.26 times higher (Figure 4). Therefore, we confirmed that HCVcc cells do have increased *CK8* expression.

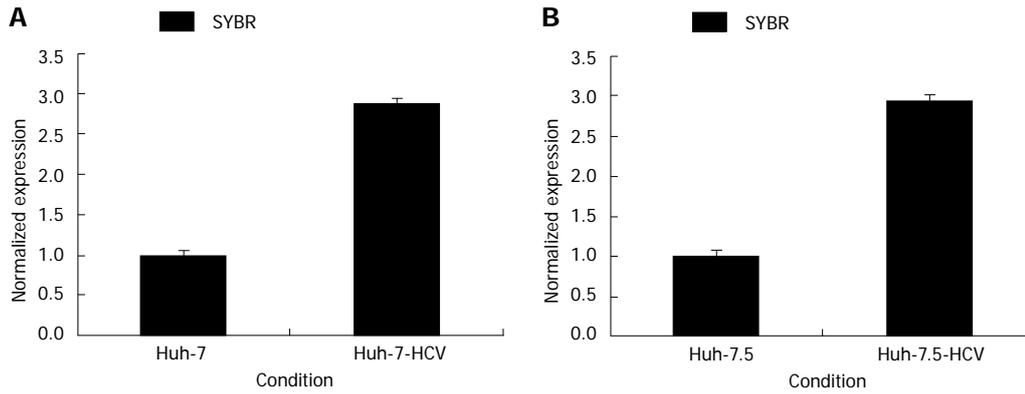


Figure 3 Relative cyokeratin 8 mRNA expression in Huh-7 and Huh-7- hepatitis C virus cells (A), or Huh-7.5 and Huh-7.5- hepatitis C virus cells (B).

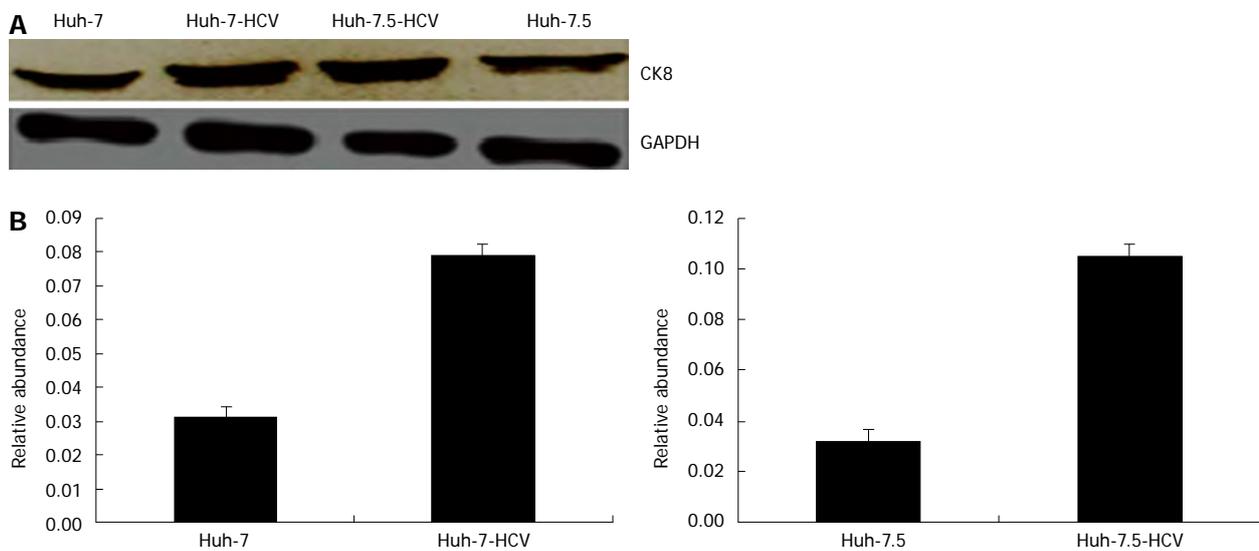


Figure 4 Cyokeratin 8 expression determined at the protein level by Western blotting in Huh-7 and Huh-7-hepatitis C virus cells (A), or Huh-7.5 and Huh-7.5-hepatitis C virus cells (B). HCV: Hepatitis C virus; CK8: Cyokeratin 8; GAPDH: Glyceraldehydes-3-phosphate dehydrogenase.

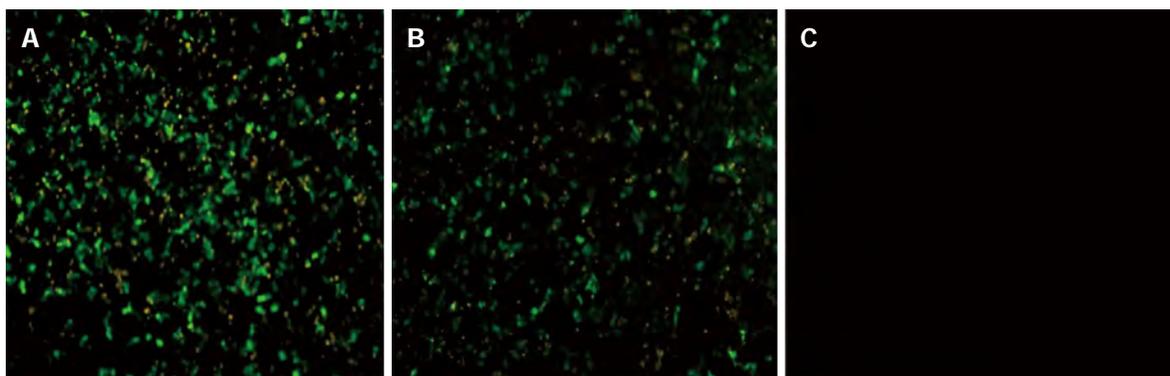


Figure 5 Inverted fluorescence microscopic observation 24 h after cyokeratin 8 transfection ( $\times 200$ ). A: SMMC7721 cells transfected by pEGFP-CK8 recombination vector; B: SMMC7721 cells transfected by pEGFP-C1 vector; C: SMMC7721 cells without transfection. CK8: Cyokeratin 8.

**Inverted fluorescence microscopic observation**

We next ectopically expressed CK8 in SMMC7721 cells. We confirmed the overexpression of CK8 in cells under inverted fluorescence microscope 24 h after transfection. Since the CK8 expression vector contains an EGFP marker, we observed that SMMC7721/pEGFP-C1 and SMMC7721/pEGFP-CK8 cells appeared bright

green compared to control SMMC7721 cells (Figure 5). This data indicated that ectopic expression of CK8 was achieved in SMMC7721 cells.

**CK8 mRNA expression by qRT-PCR**

The  $2^{-\Delta\Delta Ct}$  value of CK8 mRNA levels in SMMC7721, SMMC7721/pEGFP-C1, and SMMC7721/pEGFP-CK8

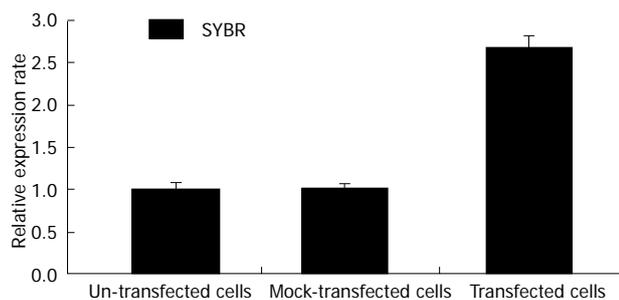


Figure 6 Cytokeratin 8 relative expression at the mRNA level.

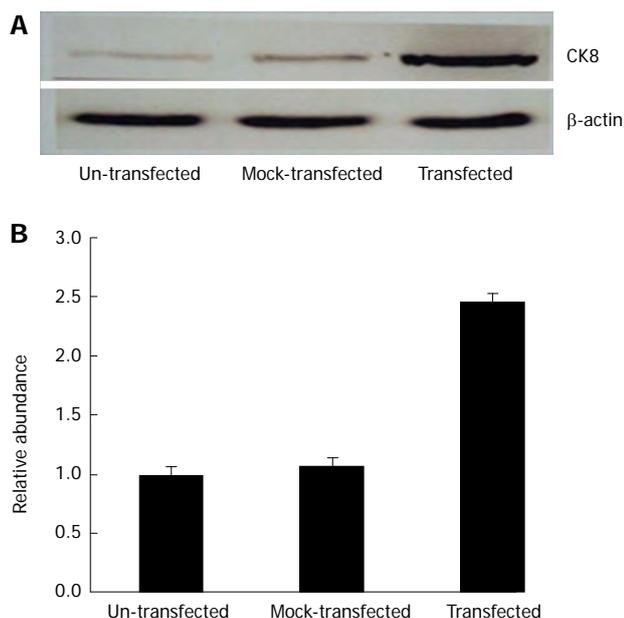


Figure 7 Cytokeratin 8 expression at the protein level 48h after transfection. CK8: Cytokeratin 8.

cells are shown in Figure 6. Beta-actin was used as the housekeeping gene, while SMMC7721 cells were used for baseline detection. The results were expressed as fold-change. *CK8* expression at the mRNA level was significantly upregulated in SMMC7721/pEGFP-*CK8* cells. *CK8* expression in SMMC7721/pEGFP-*CK8* cells was 2.69 times higher than in SMMC7721 cells, and was 2.64 times higher than in SMMC7721/pEGFP-C1 cells.

#### Ectopic expression of *CK8* determined by Western blot analysis

Using Western blotting, we compared the chemiluminescent signals of *CK8* and  $\beta$ -actin in SMMC7721, SMMC7721/pEGFP-C1, and SMMC7721/pEGFP-*CK8* cells. The ratio between *CK8* and  $\beta$ -actin were reflective changes in *CK8* expression. *CK8* expression in SMMC7721/pEGFP-*CK8* cells was 2.46 times higher than in SMMC7721 cells, and 2.29 times higher than in SMMC7721/pEGFP-C1 cells. This demonstrated that ectopic expression of *CK8* was observed at the protein level in SMMC7721/pEGFP-*CK8* cells (Figure 7). Therefore, we confirmed that *CK8* expression was increased

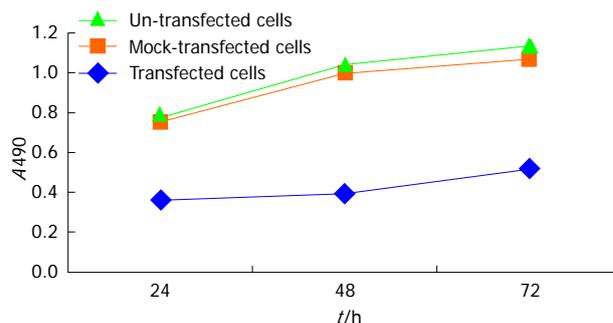


Figure 8 Growth chart after transfection in three groups of cells.

in SMMC7721 cells after transfection with pEGFP-*CK8* vector.

#### Effects of ectopic *CK8* overexpression on cell proliferation

Using MTT detection, we determined the effects of ectopic *CK8* expression on SMMC7721 cells 72 h after transfection. *CK8* overexpression decreased the growth and proliferation of SMMC7721 cells compared to control cells and mock-transfected cells (Figure 8). This data indicated that ectopic *CK8* expression decreased cell growth and proliferation of SMMC7721 cells.

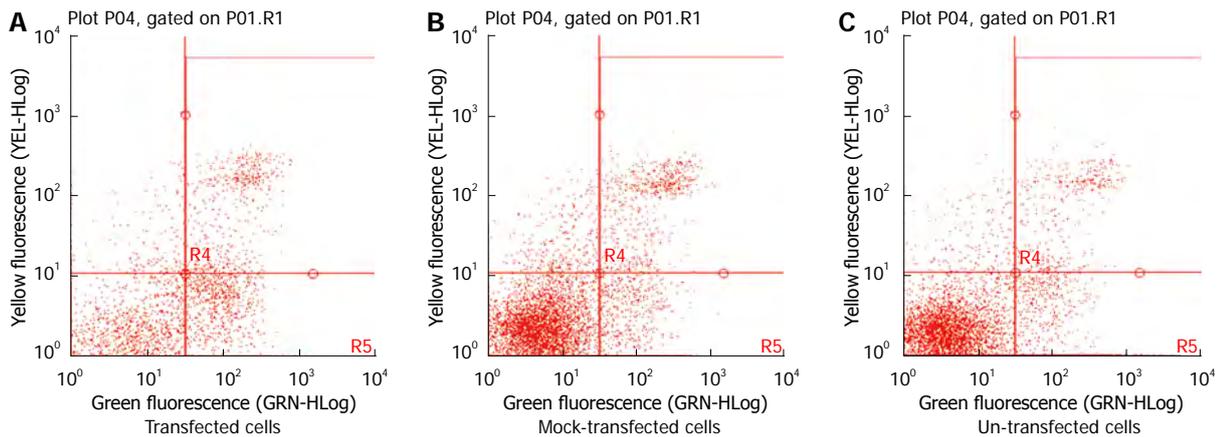
#### Effects of ectopic *CK8* expression on the apoptosis of SMMC7721 cells

We determined the effects of ectopic *CK8* expression on the apoptosis of SMMC7721 cells 48 h after transfection. Using flow cytometry, ectopic *CK8* expression increased the apoptotic rate of SMMC7721 cells, compared to untransfected and mock-transfected cells (Figure 9).

## DISCUSSION

In this study, we established a full-length HCV genomic replication in Huh-7 and Huh-7.5 cells. Lohmann *et al.*<sup>[18]</sup> reported that subgenomic HCV RNA replicons are capable of autonomously replicating in Huh7 cells. These dicistronic replicons include the neomycin-resistant gene, making them selectable by G418, and most or all of the viral nonstructural genes<sup>[19,20]</sup>. This system provides a novel and powerful tool for the study of HCV replication mechanisms and for study of the interaction between host and viral factors involved in viral progression<sup>[21,22]</sup>. In our study, we transfected Huh-7 and Huh-7.5 cells to express HCV RNA and generated the HCVcc cell line. We used qRT-PCR, immunofluorescence, and TEM to detect HCV RNA, HCV core protein, and HCV particles, respectively. The results confirmed that HCV expression in Huh-7 and Huh-7.5 cells led to the production of HCV particles.

*CK8* is a cytoskeletal intermediate filament protein that abundantly expresses in hepatocytes to maintain cell integrity, and prevent mechanical and non-mechanical cell injury<sup>[23,24]</sup>. Previous studies showed that *CK8* was upregulated in HBV-infected liver tissues from p21-HBx mice<sup>[25]</sup>,



**Figure 9 Cell flow cytometry.** A: SMMC7721/pEGFP-cytokeratin 8 cells; B: SMMC7721/pEGFP-C1 cells; C: SMMC7721 cells without transfection. Cells were collected and washed twice with PBS, suspended in 200  $\mu$ L binding buffer and 10  $\mu$ L annexin V-FITC for 20 min in the dark, and thereafter, 300  $\mu$ L binding buffer and 5  $\mu$ L propidium iodide (PI) were added to each sample. The apoptotic cells were determined using a flow cytometer by staining with Annexin V-FITC. Representative dot plots show annexin V-FITC staining in cells. Results are representative of 3 independent experiments. FITC: Fluorescein isothiocyanate; PBS: Phosphate-buffered saline.

and that its upregulation contributed to the development and progression of HCC-induced HBV. Tai DI found that *CK8* was focally positive in a patient with a malignant liver patient infected with HCV<sup>[26]</sup>. Toivola *et al*<sup>[17]</sup> found that in chronic HCV infection, *CK8* phosphorylation is a progression marker during HCV progression and regression. Furthermore, Strnad *et al*<sup>[27]</sup> found that a number of *CK8* gene variants are increased in patients with chronic HCV infection. However, it is unclear about the relation between *CK8* expression and HCVcc cells. We observed a concomitant increase in *CK8* levels, which was confirmed by RT-PCR and Western blot analysis. *CK8* mRNA expression in Huh-7-HCV and Huh-7.5-HCV cells was 2.88 and 2.95 times higher than in Huh-7 and Huh-7.5 cells, respectively. At the protein level, *CK8* expression was 2.53 and 3.26 times higher in Huh-7-HCV and Huh-7.5-HCV cells, respectively, than Huh-7 and Huh-7.5 cells. This suggests that HCV up-regulates *CK8* expression in HCVcc cells, and that *CK8* expression is significantly associated with HCV.

*CK8* plays a role in maintaining cellular structural integrity, signal transduction, and cellular differentiation<sup>[28-32]</sup>. Snider NT demonstrated that acetylation of *CK8* was up-regulated in diabetic human livers<sup>[33]</sup>. We showed that HCV up-regulates *CK8* expression in HCVcc cells. However, the biological function of ectopic *CK8* in tumor cells is not fully elucidated. To further investigate the biological function of aberrant *CK8* expression, we cloned the full length CDS of *CK8* to establish the eukaryotic expression recombination vector pEGFP-*CK8*. To study the biological function of increased *CK8* on cells independently, we chose another cell line called SMMC7721 cells in our laboratory. SMMC7721 cells were transfected by pEGFP-*CK8* recombination vector, and under an inverted fluorescence microscope we observed the expression and distribution of GFP-tagged *CK8*. In addition, by RT-PCR and Western blot analysis, we found that *CK8* mRNA levels in SMMC7721/pEGFP-*CK8* cells was 2.69 and 2.64 times higher than in SMMC7721

cells and SMMC7721/pEGFP-C1 cells, respectively. At the protein level, *CK8* expression in SMMC7721/pEGFP-*CK8* cells was 2.46 and 2.29 times higher than in SMMC7721 and SMMC7721/pEGFP-C1 cells, respectively. These observations showed that *CK8* gene was transcribed and expressed in SMMC7721 cells.

*CK8* abnormal expression and mutations can lead to acute or sub-acute liver injury and promote tumor cells apoptosis<sup>[34,35]</sup>. The persistent expression of *CK8* can induce tumor cell apoptosis through a number of transcription factors that regulate a large number of oncogenes<sup>[36]</sup>. In SMMC7721 transfected by pEGFP-*CK8*, we further observed the biological effects of increased *CK8* on cells. We detected proliferation and apoptosis by MTT reaction and flow cytometry, respectively. We found that ectopic *CK8* expression decreased cell growth and proliferation, and increased apoptosis of SMMC7721 cells. Therefore, we concluded that the abnormal expression of *CK8* regulates cellular pathological injury. However, it is unclear what the mechanisms are by which *CK8* affects cell cycle and apoptosis. In conclusion, these results suggest *CK8* up-regulation might have a functional role during HCV infection and pathogenesis, and it could be a promising target for the treatment of HCV infection.

In summary, we successfully established and identified HCVcc and observed that *CK8* is upregulated in HCVcc. Overexpression of *CK8* in SMMC7721 cells inhibited cell proliferation and induced apoptosis. *CK8* could be a potential target for the treatment of HCV infection. Future studies will (1) identify the interactions of *CK8* with other proteins to mediate its effects; (2) assess how *CK8* expression regulates a number of known oncogenes in HCV; and (3) determine how *CK8* promotes apoptosis.

## COMMENTS

### Background

Currently, several proteins have been identified to be overexpressed during hepatitis C virus (HCV) infection and pathogenesis. Studies have suggested

that cytokeratin 8 (*CK8*) is closely related to a number of liver diseases. *CK8* knock-out mice develop liver hemorrhage and are more susceptible to liver injury. However, it remains unknown whether HCV affects *CK8* levels in their established *in vitro* HCV cell culture system (HCVcc) and the biological and functional role of *CK8* in hepatoma cells.

### Research frontiers

It has been reported that there are more than 100 abnormal proteins expressed in HCV-infected cells and hepatitis C patients. Studies determining the changes in protein expression associated with HCV infection will help to elucidate host/virus interactions, and provide further insight to HCV pathogenesis. *CK8* plays a crucial role in maintaining the structural integrity and the mechanical properties of cells. Recent studies have suggested that *CK8* is involved in several liver diseases. Much interest is shown to understand *CK8* overexpression during HCV infection and to investigate the role of ectopic *CK8* expression in hepatoma cell lines.

### Innovations and breakthroughs

In this study, the authors transfected Huh-7 and Huh-7.5 cells to express HCV RNA and generated the HCVcc cell line. Previous studies showed that *CK8* is upregulated in HBV-infected liver tissues from p21-HBx mice and in a patient with a malignant liver infected with HCV. However, it is unclear what the relation between *CK8* expression and HCVcc cells is. The authors observed a concomitant increase in *CK8* levels by real-time Polymerase chain reaction and Western blot analysis. The results show that HCV up-regulates *CK8* expression in HCVcc cells. However, the biological function of ectopic *CK8* in tumor cells is not fully elucidated. The authors found that ectopic *CK8* expression decreased cell growth and proliferation, and increased apoptosis of SMMC7721 cells. Therefore, the authors concluded that the abnormal expression of *CK8* regulates cellular pathological injury.

### Applications

The results of this study suggest that *CK8* up-regulation might have a functional role during HCV infection and pathogenesis, and it could be a promising target for the treatment of HCV infection.

### Peer review

This is a very well written manuscript. In this paper, the authors show the over-expression of *CK8* in an *in vitro* HCV cell culture system. Large-scale proteome analyses of the *in vitro* HCV infection model have also been performed. Thus new hopes characterize the HCV field and new advances are reasonably expected. Here, *CK8* is found up-regulated in Huh7 and Huh7.5 cells infected with chimeric full length HCV genome. The methodology is acceptable. The conclusions are interesting.

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## Screening pre-bariatric surgery patients for esophageal disease with esophageal capsule endoscopy

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### Abstract

**AIM:** To determine if esophageal capsule endoscopy (ECE) is an adequate diagnostic alternative to esophagogastroduodenoscopy (EGD) in pre-bariatric surgery patients.

**METHODS:** We conducted a prospective pilot study to assess the diagnostic accuracy of ECE (PillCam ESO2, Given Imaging) vs conventional EGD in pre-bariatric surgery patients. Patients who were scheduled for bariatric surgery and referred for pre-operative EGD were prospectively enrolled. All patients underwent ECE followed by standard EGD. Two experienced gastroenterologists blinded to the patient's history and the findings

of the EGD reviewed the ECE and documented their findings. The gold standard was the findings on EGD.

**RESULTS:** Ten patients with an average body mass index of 50 kg/m<sup>2</sup> were enrolled and completed the study. ECE identified 11 of 14 (79%) positive esophageal/gastroesophageal junction (GEJ) findings and 14 of 17 (82%) combined esophageal and gastric findings identified on EGD. Fisher's exact test was used to compare the findings and no significant difference was found between ECE and EGD ( $P = 0.64$  for esophageal/GEJ and  $P = 0.66$  for combined esophageal and gastric findings respectively). Of the positive esophageal/GEJ findings, ECE failed to identify the following: hiatal hernia in two patients, mild esophagitis in two patients, and mild Schatzki ring in two patients. ECE was able to identify the entire esophagus in 100%, gastric cardia in 0%, gastric body in 100%, gastric antrum in 70%, pylorus in 60%, and duodenum in 0%.

**CONCLUSION:** There were no significant differences in the likelihood of identifying a positive finding using ECE compared with EGD in preoperative evaluation of bariatric patients.

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**Key words:** Capsule endoscopy; Bariatric; Moderate sedation; Esophagogastroduodenoscopy; Esophageal capsule endoscopy

**Core tip:** This is the first prospective study that shows in pre-bariatric patients, capsule endoscopy can be used to identify positive esophageal disorders when compared to a sedated esophagogastroduodenoscopy. Further studies are needed to help define the role of esophageal capsule endoscopy as a tool for pre-operative evaluation.

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## INTRODUCTION

In patients with morbid obesity, surgery is a treatment option associated with good medium and long-term results with procedures such as gastric banding, sleeve gastrectomy, gastric bypass, and biliopancreatic diversion<sup>[1]</sup>. These operations can be performed laparoscopically in most obesity centers<sup>[2-4]</sup>. Preoperative esophagogastroduodenoscopy (EGD) is useful to detect pathological findings that might preclude or delay bariatric surgery<sup>[5]</sup>.

Patients referred for bariatric surgery often have comorbidities including obstructive sleep apnea, arterial hypertension, coronary heart disease or diabetes mellitus which puts them at risk for any procedure that involves conscious sedation. The risk of EGD in these patients includes aspiration, hypoxemia, hypoventilation, airway obstruction, vasovagal episodes, and arrhythmias. Esophageal capsule endoscopy (ECE) offers a less invasive diagnostic alternative in evaluating diseases of the esophagus. ECE does not require sedation and is therefore better tolerated by patients. Several studies have shown that ECE is an adequate alternative diagnostic method for esophageal variceal screening and diagnosis of Barrett's esophagus in patients with chronic gastroesophageal reflux<sup>[6-8]</sup>. Delvaux *et al*<sup>[9]</sup> concluded that capsule endoscopy showed a moderate sensitivity and specificity in detecting esophageal diseases such as esophagitis, hiatal hernias, varices and Barrett's esophagus. They determined the overall positive predictive value of capsule endoscopy was 80%. In this study we aim to determine if capsule endoscopy is adequate in identifying specific esophageal and gastric pathology for patients undergoing bariatric surgery as compared to EGD.

## MATERIALS AND METHODS

This was a prospective pilot study. Patients from 2010 to 2012 who were scheduled to undergo bariatric surgery at University of California San Diego Medical Center and referred for pre-operative EGD were prospectively enrolled to assess the diagnostic accuracy of the ECE (PillCam ESO2, Given Imaging) *vs* conventional EGD. A total of ten patients were enrolled in the study. Patients were enrolled after Human Subjects Research Protection Committee approved consent was obtained. All patients underwent ECE followed by standard EGD performed under moderate sedation with fentanyl and versed. All patients underwent ECE followed by standard EGD performed by a single endoscopist that was video recorded.

Demographic data was collected on each patient including age, sex, weight in kilograms (kg), and body mass index (BMI) per patient's medical chart. Co-morbidities on each patient were documented including obstructive sleep apnea, coronary artery disease hypertension, type II diabetes mellitus, chronic kidney disease, and non-alcoholic fatty liver disease (NAFLD). Two experienced gastroenterologists reviewed the ECE and EGD videos and documented their findings. Both gastroenterologists were blinded to patients' history. Findings on ECE were then compared with the findings on EGD. Findings were categorical variables where 0 represented a normal finding and 1 represented an abnormal finding. Abnormal findings included esophagitis, Schatzki ring, and hiatal hernia. Fisher's exact test was used to compare the findings between the two modalities with the findings on the EGD considered the gold standard.

## RESULTS

Table 1 provides baseline demographic information and co-morbidities on each patient. The mean age was 46.2 years with the majority of patients being female (6/10). The average BMI was 50.12 kg/m<sup>2</sup> and average weight of 141.85 kg. Eight out of ten patients and nine out of ten patients suffered from obstructive sleep apnea and hypertension respectively. Forty percent of patients suffered from diabetes and 30% of patients had a diagnosis of NAFLD. Two out of ten patients had chronic kidney disease with a glomerular filtration rate of 55 and 48 mL/min/1.73 sqm.

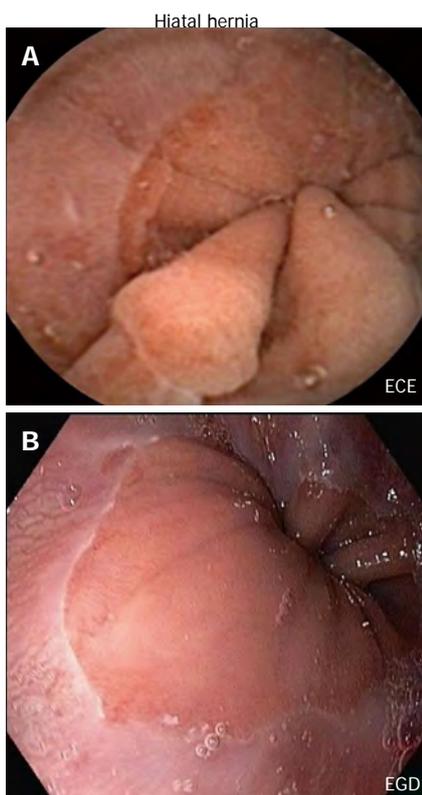
Visualization of the esophagus, GE junction, and gastric body were seen 100% of the time on ECE. The antrum and pylorus were identified 70% and 60% of the time respectively. The gastric cardia and the duodenum were not able to be identified on any capsule endoscopies. The majority of the capsules (9/10) were retained in the stomach (Table 1).

Esophageal findings on EGD included Schatzki ring, hiatal hernia and esophagitis. Two patients were noted to have gastric findings. One patient was found to have mild gastropathy and the other patient was described as having a watermelon stomach at the pylorus.

ECE identified 11 of 14 (79%) positive esophageal/gastroesophageal junction (GEJ) findings and 14 of 17 (82%) combined esophageal and gastric findings identified on EGD. Correctly identified abnormal findings on ECE as seen on EGD in the esophagus included hiatal hernia in seven out of ten patients (Figure 1), Schatzki ring in one out of three patients and felinization rings were correctly identified in one patient on both ECE and EGD. Gastric findings seen on ECE as well as EGD included gastropathy in the body of the stomach in three out of three patients. Erosions were correctly identified on ECE and EGD in one patient. There was a trend toward agreement among ECE and EGD findings in the esophagus *vs* stomach (35/40 findings in agreement in the esophagus *vs* 30/40 findings in agreement in the

**Table 1 Results of patient characteristics and visualization (n = 10) n (%)**

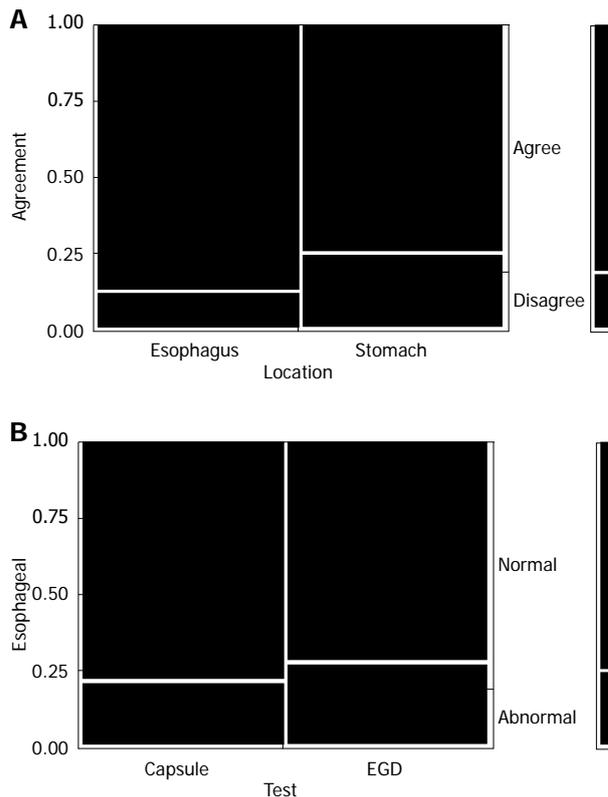
Result	Value
<b>Characteristics</b>	
Mean age (yr) (median)	46.2 (46)
Female	6 (60)
Mean body mass index (kg/m <sup>2</sup> ) (median)	50.12 (48.1)
Weight (kg) (median)	141.85 (139.9)
Obstructive sleep apnea	8 (80)
Coronary artery disease	1 (10)
Hypertension	9 (90)
Diabetes mellitus	4 (40)
Chronic kidney disease	2 (20)
Non-alcoholic fatty liver disease	3 (30)
<b>Visualization</b>	
Mean esophageal transit time (min) (median)	3.997 (3.997)
Esophagus	10 (100)
Gastroesophageal junction	10 (100)
Gastric cardia	0 (0)
Gastric body	10 (100)
Gastric antrum	7 (70)
Pylorus	6 (60)
Duodenum	0 (0)



**Figure 1 Comparison of esophageal capsule endoscopy vs esophagogastroduodenoscopy.** Image of a hiatal hernia on esophageal capsule endoscopy (ECE, panel A) vs esophagogastroduodenoscopy (EGD, panel B). ECE was able to correctly abnormal findings in the esophagus such as hiatal hernias when compared to EGD.

stomach) (Figure 2A).

Fisher's exact test was used to compare the findings and no significant difference was found between ECE and EGD ( $P = 0.64$  for esophageal/GEJ and  $P = 0.66$  for combined esophageal and gastric findings respec-



**Figure 2 Inter-observer agreement between esophageal and gastric findings and results of esophageal findings on capsule vs esophagogastroduodenoscopy.** A: There was a trend toward agreement among esophageal capsule endoscopy and esophagogastroduodenoscopy findings in the esophagus vs stomach (35/40 findings in agreement in the esophagus vs 30/40 findings in agreement in the stomach); B: There was no significant difference between esophageal capsule endoscopy and esophagogastroduodenoscopy (EGD) ( $P = 0.64$ ) for combined esophageal and gastroesophageal junction findings on 2-tailed Fischer exact test ( $P = 0.66$ ).

tively) (Figure 2B). Of the positive esophageal/GEJ findings, ECE failed to identify the following: hiatal hernia in two patients, mild esophagitis in two patients, and mild Schatzki ring in two patients. Conversely, ECE identified a Schatzki ring in one patient, an irregular Z-line in another patient, and a hiatal hernia in a third patient not identified on EGD. There were no adverse events related to ECE.

## DISCUSSION

Bariatric surgery is increasingly performed to treat morbid obesity and its complications. Pre-operative EGD can help identify useful pathology that can negatively influence post-operative outcomes. However EGD is an invasive procedure requiring the use of conscious sedation that can carry risks of cardiopulmonary complications, hypoxemia, aspiration and cardiac arrhythmias<sup>[10-13]</sup>. Quine *et al*<sup>[10]</sup> showed in a prospective study of 14149 upper endoscopies that 31 patients experienced cardiopulmonary complications related to moderate sedation. Similar studies have shown the rate of cardiopulmonary complications to range from 1.3 per thousand to 5.4 per thousand<sup>[11,14]</sup>. Sharma *et al*<sup>[15]</sup> showed that the risk of developing unplanned cardiopulmonary events following

upper endoscopy was 0.6%. Multiple studies have determined that the risks of moderate sedation in patients undergoing endoscopy increase with co-morbidities such as advanced age, obstructive sleep apnea, underlying heart disease, and obesity<sup>[10-12,14,16]</sup>. Sharma *et al*<sup>[15]</sup> found that an increased American Society of Anesthesiologists classification (ASA class score) was attributed to an increase risk of developing cardiopulmonary complications. Odds ratio ranged from 1.1 with an ASA II score to 1.8 with ASA class III and 7.4 with class V. Many bariatric surgery patients will generally have higher ASA scores.

Endoscopic capsule was initially approved by the Food and Drug Administration in 2001 for evaluation of the small bowel. It is increasingly being used for the evaluation of obscure GI bleeding, Crohns' disease, and suspected small bowel tumors<sup>[17-21]</sup>. Several studies have shown that capsule endoscopy can be used to identify esophageal disorders such as Barrett's esophagus/esophagitis, hiatal hernia, and esophageal varices<sup>[22-26]</sup>.

Our study shows that in pre-bariatric patients, capsule endoscopy can be used to identify positive esophageal disorders when compared to a sedated EGD. Capsule endoscopy was correctly able to identify hiatal hernias, Schatzki rings and esophagitis when compared with EGD. However in our study ECE failed to identify gastric pathology the majority of the time. The gastric cardia was not visualized during ECE and the gastric antrum and pylorus were identified 30% and 40% of the time respectively. The duodenum also could not be correctly identified by ECE due to the short capsule recording time of 20 min.

Esophageal findings seen on ECE but not noted on EGD included a Schatzki ring in one patient, an irregular Z-line in another patient, and a hiatal hernia in a third patient. This might be explained by changes in body position between the upright ECE and left lateral decubitus EGD.

Further limitations of this pilot study include a small patient size. Only ten patients were enrolled in the trial. Despite this, results trended towards no significant difference in the likelihood of identifying a positive finding using ECE compared with EGD.

In conclusion, this is the first study to suggest that ECE may be a safer alternative than sedated EGD for evaluation of esophageal disorders prior to bariatric surgery. However ECE cannot consistently evaluate for gastric or duodenal pathology. Further studies are needed to help define the role of ECE as a tool for pre-operative evaluation.

## COMMENTS

### Background

In patients with morbid obesity, bariatric surgery is a treatment option associated with good medium and long-term results. Prior to surgery, many patients undergo an endoscopic examination to determine if they have any pathology to prevent or delay them from undergoing bariatric surgery. Esophagogastroduodenoscopy (EGD) however is an invasive procedure which comes with risks including the risk of sedation associated with morbidly obese patients. Small bowel capsule endoscopy has been used to identify pathology in the small

bowel for years. Esophageal capsule endoscopy (ECE) uses the same technology to examine the esophagus and stomach. Several studies have shown that ECE is able to identify pathology and anatomic variations in the esophagus and stomach, and can be performed without the risks of moderate sedation. No studies to date have been done to determine whether capsule endoscopy is equivalent to EGD in screening pre-bariatric surgery patients.

### Research frontiers

Small bowel capsule endoscopy was developed as a means to non-invasively examine the small bowel for pathology. ECE was subsequently developed and food and drug administration approved using the same technology to examine the esophagus and stomach. There have been several studies to suggest that ECE is comparable to EGD in the diagnosis of upper gastrointestinal disorders such as Barrett's esophagus and esophageal varices.

### Innovations and breakthroughs

Small bowel capsule endoscopy is a useful tool to non-invasively examine the small bowel. ECE uses the same technology to examine the esophagus and stomach. Recent studies have shown that ECE is comparable EGD in diagnosing esophageal pathology such as esophagitis, Barrett's esophagus and esophageal varices as well as stomach pathology such as gastric cancers. However ECE has never been compared to EGD in pre-screening patients for pathology which may preclude them from bariatric surgery. This study is the first study to date to compare the two in this patient population.

### Applications

The study results indicate that ECE may be a safer alternative than sedated EGD for evaluation of esophageal disorders prior to bariatric surgery, but cannot consistently evaluate for gastric or duodenal pathology. Further studies are needed to help define the role of ECE as a tool for pre-operative evaluation.

### Terminology

Bariatric surgery: Surgery is performed on the stomach and/or intestines in order to facilitate weight loss in obese patients. This could be achieved either through restrictive alone or both restrictive and malabsorptive mechanisms. EGD: An imaging test that involves visually examining the lining of the esophagus, stomach, and upper duodenum with a flexible fiberoptic endoscope; ECE: A small camera inside a capsule shaped and sized like a pill which is used to take video images of the digestive tract to help in evaluation of symptoms such as gastrointestinal bleeding or abdominal pain; Moderate sedation: Drug induced consciousness (typically carried out with a combination of a narcotic such as fentanyl and benzodiazepine such as midazolam) during which patients respond purposefully to verbal commands, either alone or by light tactile stimulation. No interventions are needed to maintain a patent airway, patient is able to spontaneously breathe during moderate sedation. Used in a wide variety of medical procedures.

### Peer review

The results suggest that ECE may be a safer alternative than sedated EGD for evaluation of esophageal disorders prior to bariatric surgery. The paper would be desirable to specify

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## Acid and non-acid reflux in patients refractory to proton pump inhibitor therapy: Is gastroparesis a factor?

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### Abstract

**AIM:** To determine whether an increased number and duration of non-acid reflux events as measured using the multichannel intraluminal impedance pH (MII-pH) is linked to gastroparesis (GP).

**METHODS:** A case control study was conducted in which 42 patients undergoing clinical evaluation for continued symptoms of gastroesophageal reflux disease (both typical and atypical symptoms) despite acid suppression therapy. MII-pH technology was used over 24 h to detect reflux episodes and record patients' symptoms. Parameters evaluated in patients with documented GP and controls without GP by scintigraphy included total, upright, and supine number of acid and non-acid reflux episodes (pH < 4 and pH > 4, respectively), the duration of acid and non-acid reflux in a 24-h period, and the

number of reflux episodes lasting longer than 5 min.

**RESULTS:** No statistical difference was seen between the patients with GP and controls with respect to the total number or duration of acid reflux events, total number and duration of non-acid reflux events or the duration of longest reflux episodes. The number of non-acid reflux episodes with a pH > 7 was higher in subjects with GP than in controls. In addition, acid reflux episodes were more prolonged (lasting longer than 5 min) in the GP patients than in controls; however, these values did not reach statistical significance. Thirty-five patients had recorded symptoms during the 24 h study and of the 35 subjects, only 9% ( $n = 3$ ) had a positive symptom association probability (SAP) for acid/non-acid reflux and 91% had a negative SAP.

**CONCLUSION:** The evaluation of patients with a documented history of GP did not show an association between GP and more frequent episodes of non-acid reflux based on MII-pH testing.

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**Key words:** Gastroparesis; Non-acid gastroesophageal reflux; Acid gastroesophageal reflux; Multi-channel intraluminal impedance; Functional bowel disorder

**Core tip:** Gastroparesis (GP) has been thought to occur in about 8%-10% of patients who suffer from refractory gastroesophageal reflux disease (GERD). There have been no formal studies to date that have evaluated whether patients with refractory GERD additionally suffer from GP. Our study aimed to investigate whether patients who experience continued symptoms of GERD despite acid suppression therapy also concurrently have gastroparesis. By using multichannel intraluminal impedance pH technology, we were not able to find an association between patients with refractory GERD and gastroparesis.

Tavakkoli A, Sayed BA, Talley NJ, Moshiree B. Acid and non-acid reflux in patients refractory to proton pump inhibitor therapy: Is gastroparesis a factor? *World J Gastroenterol* 2013; 19(37): 6193-6198 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6193.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6193>

## INTRODUCTION

Gastroparesis (GP) is a chronic gastrointestinal (GI) motility disorder characterized by delayed gastric emptying in the absence of mechanical outlet obstruction<sup>[1]</sup>. The most common etiologies of GP include those secondary to chronic disease states such as diabetes mellitus and collagen-vascular diseases, post-surgical causes, medication induced, and idiopathic causes<sup>[1,2]</sup>. Symptoms of GP include early satiety, nausea, vomiting, abdominal pain, bloating, and gastroesophageal reflux<sup>[1-3]</sup>. Notably, GP has been rising in prevalence across the United States, which as a result, has led to an increase in GP related hospitalizations and increasing health care costs<sup>[1,4]</sup>.

Gastroesophageal reflux disease (GERD) is a very common disease in the United States and has recently been shown to be the most common GI diagnosis among clinical visits in the United States<sup>[5]</sup>. The mainstay of treatment for GERD includes proton pump inhibitors (PPIs) but several studies have shown that upwards of 40% of patients who suffer from heartburn have either a partial or complete lack of response to PPIs taken once a day<sup>[6-8]</sup>. A number of causes for PPI failure have been investigated, including medication non-compliance, undiagnosed functional bowel disorders, and GP<sup>[9]</sup>.

GP has been reported to be present in a variable subset of patients who suffer from refractory GERD and studies have shown that anywhere between 8%-10% of patients with refractory GERD additionally suffer from GP<sup>[10-14]</sup>. It is unknown whether the hypothesized link between GP and refractory GERD actually exists in clinical practice. The aim of our study was to determine whether there is an increased number and duration of non-acid reflux events when comparing patients with GP and without GP using multichannel intraluminal impedance pH (MII-pH). Given the delayed gastric emptying of food in patients with GP, we hypothesized that patients with GP will experience a greater number of non-acid reflux episodes for a longer duration of time when compared to controls without GP.

## MATERIALS AND METHODS

The University of Florida Health Science Center Institutional Review Board approved the collection and analysis of MII-pH data obtained in this study.

### Patient population

From July 2009 to September 2010, all patients who underwent MII-pH analysis for continued symptoms of

GERD despite PPI therapy were enrolled after signing informed consent ( $n = 66$ ). The patients' health records were reviewed for evidence of a prior gastric emptying scintigraphy (GES) done at the University of Florida ( $n = 39$ ) or the results were documented in a recent clinic note from a prior GES done at an outside institution ( $n = 3$ ). Patients were then included in this study if a GES was previously done (abnormal at University of Florida if  $t^{1/2} > 90$  min) and they were scheduled to or had undergone a MII-pH study at the gastric motility laboratory at University of Florida. Patients were excluded from the study if they had no prior GES or history of one documented in a recent clinical note. However, patients were not excluded if they were currently on anti-acid therapy. All patients underwent a physical examination and upper endoscopy prior to undergoing the ambulatory MII-pH testing to exclude any mechanical obstruction.

Patients were considered refractory to PPI therapy at the discretion of the clinician who had referred them to receive MII-pH analysis. Patients were defined as "refractory" if they continued to have symptoms of GERD despite anti-acid therapy. All patients remained on their previously prescribed PPI therapy during the duration of the study.

### MII-pH monitoring

Subjects presented to the motility laboratory after an overnight fast. The Sleuth ambulatory system (Sandhill Scientific, Inc; Highlands Ranch, CO, United States) was used to perform the impedance testing. The catheter has an antimony pH electrode measuring the pH 5 cm from the distal tip and 6 impedance electrodes that measure impedance at 3, 5, 7, 9, 15 and 17 cm above the distal tip. The catheter was placed transnasally with its tip 5 cm above the lower esophageal sphincter.

Subjects then underwent a 24-h monitoring in which their symptoms were electronically recorded and the patients kept a diary of their food intake. All 42 subjects were continued on their current acid suppression therapy for the entire duration of the study. All prokinetic agents, including bethanechol, domperidone, metoclopramide, and azithromycin were discontinued.

Symptoms recorded in the electrical diaries included both typical GERD symptoms (heartburn, regurgitation, and chest pain) and atypical GERD symptoms (cough, hoarseness, abdominal discomfort, nausea, belching, globus sensation, and dysphagia). After the 24-h ambulatory period, subjects returned to the motility laboratory where the data was transferred and analyzed using a single investigatory dedicated software (BioView Analysis; Sandhill Scientific, Inc.). Two investigators in the motility laboratory manually reviewed tracings and electronic diary entry of symptoms. Meal periods were marked and excluded from the analysis. Only liquid and mixed liquid/gas reflux episodes were evaluated for this study.

The parameters obtained from the MII-pH device included the total, upright, and supine number of acid and non-acid reflux episodes, the duration of acid and non-

**Table 1** Demographics and esophagogastroduodenoscopy results among patients with gastroparesis and controls

Result	GP	Controls	P value
Demographics			
Age (mean $\pm$ SD, yr)	54 $\pm$ 14	51 $\pm$ 13	0.620
Female (n)	12	19	
Males (n)	4	7	
Gastric emptying scintigraphy $t^{1/2}$ time (mean $\pm$ SD, min)	134 $\pm$ 59	65 $\pm$ 13	0.0003
Comorbidities (n)			
GERD	16	26	
Irritable bowel syndrome	2	1	
Diabetes mellitus	1	2	
Hypothyroidism	1	4	
Chronic constipation	3	2	
Hepatitis C	1	1	
IBD	1	0	
Previous surgical procedures (n)			
Cholecystectomy	4	6	
Nissen Fundoplication	3	1	
EGD status (n)			
Normal EGD	6	11	
Gastritis ( <i>H. pylori</i> negative)	7	6	
Atrophic gastritis	1	0	
Fundic gland polyp	2	0	
Hyperplastic polyp	0	1	
Antacid usage (n)			
H2 blocker therapy	2	5	
Daily	1	3	
<i>Bid</i>	1	1	
<i>Tid</i>	0	1	
PPI therapy (n)	14	15	
Daily	4	4	
<i>Bid</i>	9	10	
<i>Tid</i>	1	1	
Sucralfate (n)	2	3	
Antacids (n)	1	1	
No therapy (n)	0	3	

EGD: Esophagogastroduodenoscopy; GERD: Gastroesophageal reflux disease; IBD: Inflammatory bowel disease; *H. pylori*: *Helicobacter pylori*; PPI: Proton pump inhibitor.

acid reflux in a 24-h period, and the number of reflux episodes lasting longer than 5 min. The MII-pH detected reflux episodes that were classified as acidic when the esophageal pH fell below 4, non-acidic if the pH was between 4-7, and alkaline if the pH rose above 7.

Based on the MII-pH data, we evaluated each separate symptom and determined their association with a reflux episode. The symptom association probability (SAP) was electronically calculated using the BioView Analysis Software. The 24-h pH data was divided into 2-min segments and each of the 2 min segments were studied to determine whether a reflux event and a symptom occurred during that segment. A 2  $\times$  2 table was made in which the number of two minute segments with and without reflux and with and without symptoms were tabulated. A  $\chi^2$  test was then used to determine whether the occurrence of the symptoms and reflux could have occurred by chance. The SAP was then calculated using the formula  $SAP = (1 - P) \times 100\%$  and it was considered positive if  $> 95\%$ <sup>[15]</sup>.

### Statistical analysis

The impedance pH data was compared using the Wilcoxon non-parametric analysis of variance for means and the 2-sided Fisher exact test for proportions. All values are expressed as a mean  $\pm$  SD. The null hypothesis assumes that no significant difference in the number of non-acid reflux events will be seen when comparing impedance pH data between the GP group and the control group.

## RESULTS

### Demographics and medical conditions

The study included 42 participants chosen based on their GES results and then divided into two groups: subjects with gastroparesis and subjects with normal GES both undergoing MII-pH for continued symptoms of GERD despite PPI therapy.

The GP group included 16 patients with a mean age of 54  $\pm$  14 years (age range: 24-76 years) with a mean GES half-time ( $t^{1/2}$ ) of 134  $\pm$  59 min (normal defined at UF as  $t^{1/2}$  between 45-90 min). The control group consisted of 26 patients with a mean age of 51  $\pm$  13 years (age range: 24-77 years) with a mean GES ( $t^{1/2}$ ) of 65  $\pm$  13 min (Table 1). Among the GP group, 6 patients had a normal esophagogastroduodenoscopy (EGD) and 7 patients had biopsy proven *Helicobacter pylori* (*H. pylori*) negative gastritis (Table 1). In the control group, 11 patients had a normal EGD and 6 patients had biopsy proven *H. pylori* negative gastritis (Table 1).

Symptoms on presentation among both the GP and control group included heartburn, bloating, nausea/vomiting chest pain, and hoarseness. Concurrent medical diagnoses included GERD, irritable bowel syndrome, type II diabetes mellitus, and chronic constipation. Prior surgical procedures in both populations included cholecystectomy and Nissen fundoplication.

### MI-pH data

No statistical difference was seen between subjects with GP and controls with respect to the total number and duration of acid reflux events [13.3  $\pm$  17.1 (95%CI: 4.2-22.4) in GP *vs* 12.0  $\pm$  14.8 (95%CI: 6.0-18.0) in controls,  $P < 0.79$ ], total number and duration of non-acid reflux events [21.6  $\pm$  24.6 (95%CI: 8.5-34.7) in GP *vs* 25.7  $\pm$  29.3 (95%CI: 13.9-37.5) in controls,  $P < 0.64$ ], or the total number and duration of reflux events [30.8  $\pm$  36.5 (95%CI: 11.3-50.2) in GP *vs* 37.9  $\pm$  35.7 (95%CI: 23.48-52.29) in controls,  $P < 0.54$ ] (Figure 1). The number of non-acid reflux episodes with a pH  $> 7$  were higher in subjects with GP [5.3  $\pm$  5 (95%CI: 2.6-8.0) *vs* 4.5  $\pm$  5.6 (95%CI: 2.3-6.9) in controls,  $P < 0.67$ ] and the acid reflux episodes were more prolonged (lasting longer than 5 min) in the GP group [0.95  $\pm$  2.0 (95%CI: -1.1-2.0) *vs* 0.25  $\pm$  0.7 (95%CI: -1.1-0.5) in controls], but these values did not reach statistical significance ( $P < 0.12$ ) (Figure 1).

### Symptom association probability

Of the 42 subjects who were evaluated, 35 subjects (83%)

**Table 2** Symptom association probability for patients with gastroparesis and controls

MII-pH symptoms	Gastroparesis (n)	Controls (n)	Positive SAP (n)	Negative SAP (n)
Chest pain	3	5	1	7
Nausea/vomiting	2	4	1	6
Regurgitation	3	4	0	14
Hoarseness	0	4	0	6
Heartburn	5	9	0	4
Globus sensation	2	2	0	4
Dysphagia	4	4	1	7
Cough	3	7	1	9
Abdominal pain	7	3	0	10
Belching	6	8	0	14
Bloating	0	2	0	2

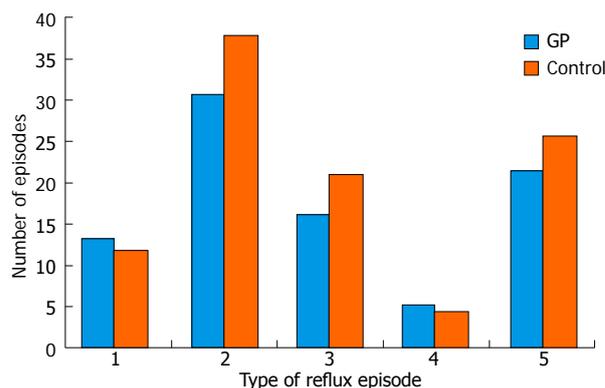
SAP: Symptom association probability; MII-pH: Multichannel intraluminal impedance pH.

recorded symptoms during the 24-h study period and 7 patients did not have any recorded symptoms. There were 87 total symptoms recorded by the 35 subjects and 33% were typical symptoms and 67% were atypical symptoms of GERD. The GP group accounted for 38% ( $n = 11$ ) of the total typical symptoms reported and the control group accounted for 62% ( $n = 18$ ) of typical symptoms. Atypical symptoms of GERD were also more commonly recorded in the control group than the GP group (59% *vs* 41% respectively) (Table 2).

Of the 35 subjects who had recorded symptoms during their MII-pH testing, only 9% ( $n = 3$ ) had a positive SAP for acid/non-acid reflux and 91% ( $n = 32$ ) had a negative SAP. Similarly, of the total typical symptoms that were recorded, 7% ( $n = 2$ ) had a positive SAP and 93% ( $n = 27$ ) had a negative SAP. Of the 58 atypical symptoms recorded, 3% ( $n = 2$ ) had a positive SAP and 97% ( $n = 56$ ) had a negative SAP. Among the 16 subjects with GP, a total of 35 symptoms were recorded and all had a negative SAP. Among the 26 controls, 52 symptoms were recorded, and 8% of those had a positive SAP with the majority (92%) having a negative SAP.

## DISCUSSION

Resistance to acid suppression therapy such as PPIs is the most common presentation of GERD in the tertiary care GI practices<sup>[16]</sup>. A survey of GERD patients receiving PPI therapy shows that 25%-42% of patients are refractory to a once-daily PPI dose, of which only 25% would respond to an increase in PPI dosing to twice daily<sup>[17,18]</sup>. In addition, 42% of GERD patients surveyed are dissatisfied with their PPI treatment outcomes<sup>[19]</sup>. GP has long been thought of as a risk factor for refractory GERD due to the impaired gastric accommodation, delayed gastric emptying and the subsequent loss of lower esophageal sphincter tone. Furthermore, as our study shows, symptoms of GP and GERD often overlap as



**Figure 1** Comparison of the number of reflux episodes for subjects with gastroparesis and controls. 1: Total reflux events; 2: Total acid reflux events; 3: Total non-acid reflux events; 4: Non-acid reflux events pH 4-7; 5: Non-acid reflux events pH > 7. GP: Gastroparesis.

both patients can complain of epigastric pain, abdominal bloating, nausea, and vomiting making it difficult to distinguish between the two disease processes. Given this observed overlap of symptoms, our study aimed to determine whether patients with GP and concurrent symptoms of reflux despite concurrent PPI therapy have an increased frequency and duration of non-acid reflux using impedance pH technology, as compared to those with normal gastric emptying.

Our results indicate that the total number and duration of acid, non-acid, and total reflux events was similar in GP and non-GP cases. While the number of alkaline events was slightly higher in the GP group, these alkaline reflux events represented a small percentage of the total non-acid reflux events observed (20%). Moreover, among the 35 patients who recorded symptoms during the 24 h MII-pH study, only 3 patients had a positive SAP for acid/non-acid reflux. Our findings do not support some prior studies indicating up to 40%-50% of patients with GERD have GP<sup>[12,20]</sup>, and we believe that GP likely accounts for a small percentage of refractory GERD cases than previously evaluated based on conventional pH testing. Our findings based on MII-pH testing indicate that neither acid nor non-acid reflux occur more frequently in patients with GP than those without.

Since only 3 of 35 patients had a positive SAP for acid and non-acid reflux, and given our lack of statistical correlation between GP and acid or non-acid reflux, other causes of refractory GERD need to be explored to explain patients' persistent symptoms. Notably, even the control group with refractory GERD symptoms had a poor symptom correlation with acid and non-acid reflux events. One possible explanation may be the presence of esophageal hypersensitivity, which has been proposed previously as an underlying mechanism in this patient population<sup>[16,18,21]</sup>. Esophageal distention occurs due to increased reflux volume exposing the esophagus to acidic/non-acid components of the refluxate, which in turn leads to persistent impairment of esophageal mucosa and thus results in esophageal hypersensitivity<sup>[21]</sup>. In our study, 67% of the reflux events recorded were non-acid with no

correlation to patients' symptoms, making esophageal hypersensitivity a plausible explanation for their continued symptoms.

Our study has some limitations. First, this study was limited to one tertiary care center and the small sample size ( $n = 42$  total with 16 subjects with GP) compromised the overall generalizability of the study, leading to a statistical type II error. This may account for our negative findings indicating a lack of difference in acid and non-acid reflux in patients with and without GP. Based on the mean number of acid reflux events in GP and control groups ( $P = 0.09$ ), the statistical power in the current study is 8%. *Post hoc* power analysis suggests that to achieve 90% statistical power, a sample size of 2592 for each group is necessary. In addition, the GES testing done was not based on the current national standard protocol for obtaining GES and some of the GES studies were not obtained at our institution therefore the accuracy of the measurements may influence our results<sup>[22]</sup>. This lack of uniformity of GES testing across multiple centers introduces a potential area for error, as patients with or without GP might have been misdiagnosed given lack of standardization. Furthermore, performing the MII-pH study on PPI therapy, as done in our study, may have affected our results in terms of delaying gastric emptying as shown in several studies which could lead to a type II error. By delaying acid-dependent peptic activity, PPIs impair hydrolytic digestion and therefore delay gastric emptying<sup>[23]</sup>. This finding may have clinical implications in the management of GERD in our subjects. Future studies evaluating the effect of GP on acid and non-acid reflux should be done with subjects strictly off PPI therapy 5 d prior to testing. Another limitation is that not all patients were maxed out on PPI therapy prior to the MII-pH study, making continued reflux a potential cause of their continued symptoms. Lastly, GES and MII-pH studies were completed on separate days and in most cases months apart and as a considerable intra-individual variability exists with gastric motility, this may also have contributed to achieving the negative results seen in this study<sup>[22]</sup>.

Our study has several important strengths. This is the first study evaluating whether an association exists between GP and non-acid reflux analyzed by MII-pH monitoring. While GP may have been thought to be associated with refractory GERD, studies have not validated this finding using MII-pH to diagnose acid and non-acid reflux. In addition, the idea that delayed gastric emptying is associated with GERD has been the basis of using prokinetic drugs for the treatment of GERD<sup>[20]</sup>. Considering our results, it might not be necessary to start these patients on prokinetic agents in addition to acid suppression therapy, unless evidence of esophageal dysmotility is found on esophageal manometry testing, given these drugs have significant side effects and drug-drug interactions. Other medications that improve LES pressure or decrease transient relaxations of the lower esophageal sphincter may be more beneficial for patients with any

type of reflux, acid or nonacid, than perhaps improving their gastric emptying. Moreover, our study illustrates that patients with weakly acidic and alkaline reflux likely suffer from esophageal hypersensitivity rather than continued reflux or GP as the main cause of their typical and atypical GERD symptoms. Finally, our study justifies a larger study in which a population of patients with refractory GERD off PPI therapy is evaluated for GP in close proximity to their initial MII-pH analysis combined with manometric esophageal testing. A larger study using the MII-pH as the gold standard for diagnosing non-acid reflux would better delineate whether GP and non-acid reflux are clearly associated.

In conclusion, whether gastroparesis contributes to refractory reflux remains to be established. However, based on our pilot study, a clear relationship does not exist but further studies using larger populations of patients undergoing impedance testing for refractory reflux would help to delineate this relationship.

## COMMENTS

### Background

Up to 40% of patients with gastroesophageal reflux disease (GERD) have either a partial or incomplete response to proton-pump inhibitors. Gastroparesis has been hypothesized to be a potential cause of refractory GERD in a subset of patients.

### Research frontiers

In a case-control study, 42 patients undergoing clinical evaluation for continued heartburn and regurgitation despite acid suppression therapy were evaluated with multi-channel intraluminal impedance pH monitoring and for evidence of gastroparesis.

### Innovations and breakthroughs

Their results did not show a difference between acid and non-acid reflux events among patients with gastroparesis and those without the disease process.

### Applications

While a clear relationship between gastroparesis and refractory GERD was not shown in their study, the results could be used to conduct further studies using larger populations of patients to help further delineate the relationship.

### Terminology

Multi-channel intraluminal impedance pH monitoring is a new technology that can detect intraluminal bolus movement without radiation. When it is combined with pH testing, it can detect both acid and non-acid reflux.

### Peer review

The current study is a relevant paper dealing with a difficult to treat and often poorly assessed patient population.

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## SEMS vs cSEMS in duodenal and small bowel obstruction: High risk of migration in the covered stent group

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**RESULTS:** Thirty-two SEMS were implanted in 20 patients. In all patients, endoscopic stent implantation was successful. Stent migration was observed in 9 of 16 cSEMS (56%) in comparison to 0/16 SEMS (0%) implantations ( $P = 0.002$ ). Stent overgrowth did not significantly differ between the two stent types (SEMS: 3/16, 19%; cSEMS: 2/16, 13%). One cSEMS dislodged and had to be recovered from the jejunum by way of laparotomy. Time until migration between SEMS and cSEMS in patients with and without concomitant biliary stents did not significantly differ (HR = 1.530, 95%CI 0.731-6.306;  $P = 0.556$ ). The mean follow-up was  $57 \pm 71$  d (range: 1-275 d).

**CONCLUSION:** SEMS and cSEMS placement is safe in small bowel tumor obstruction. However, cSEMS is accompanied with a high rate of migration in comparison to uncovered SEMS.

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**Key words:** Endoscopy; Digestive system; Intestinal neoplasms; Self-expandable metal stents; Tumor obstruction; Self-expandable metal stents complications

**Core tip:** Gastrointestinal obstruction is a complication of advanced cancer disease. It heavily impacts on patients' general condition. Endoscopic implantation of self-expanding metal stents (SEMS) is a safe and established procedure for palliative treatment of tumor obstruction. Covered SEMS are considered favorable concerning reobstruction by inhibiting tumor ingrowth. In contrast, uncovered SEMS might harbor a lower risk of migration and dislocation. In the present study covered SEMS were retrospectively compared with uncovered SEMS in patients with small bowel or duodenal obstruction. Significantly higher migration rates were observed in the covered SEMS group without observing significant increase of the rate of patients with tumor ingrowth indicating that uncovered SEMS should be

### Abstract

**AIM:** To compare clinical success and complications of uncovered self-expanding metal stents (SEMS) vs covered SEMS (cSEMS) in obstruction of the small bowel.

**METHODS:** Technical success, complications and outcome of endoscopic SEMS or cSEMS placement in tumor related obstruction of the duodenum or jejunum were retrospectively assessed. The primary end points were rates of stent migration and overgrowth. Secondary end points were the effect of concomitant biliary drainage on migration rate and overall survival. The data was analyzed according to the Strengthening the Reporting of Observational Studies in Epidemiology guidelines.

avored for palliative treatment of tumor obstruction of the duodenum or the small bowel.

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## INTRODUCTION

Endoscopic placement of self-expandable metal stents (SEMSs) has become a broadly accepted first line treatment option for patients with advanced malignant intestinal stenosis. Reconstitution of the intestinal transit is paramount in palliation of tumor obstruction, and endoscopic SEMS insertion is weighed against surgical intervention in terms of clinical relief, complication rate, and length of hospital stay of the patient. In the small bowel, duodenal tumor obstruction has increasingly been treated by SEMS placement<sup>[1,2]</sup>, but SEMS insertion in the lower small bowel is less often performed<sup>[3,4]</sup>. Duodenal SEMS insertion is technically feasible in more than 90% of interventions; it is safe and comes along with a faster start of oral intake, shorter length of hospital stay, lower morbidity and probably reduced in-hospital mortality as compared to surgical treatment<sup>[5-13]</sup>. SEMS may as well be preferred over surgical treatment in the lower small bowel and at anastomotic small bowel obstructions in case of recurrent malignancy<sup>[4]</sup>.

Tumor in- or over-growth can limit long-term outcome of SEMS in 12% to 21% of cases, but stent occlusion might be reduced by covering the SEMS with silicone or plastic membranes (covered SEMS, cSEMS)<sup>[1,2]</sup>.

Aim of this study was to compare outcome of SEMS vs cSEMS in small bowel tumor obstruction and to identify technical feasibility, safety, clinical impact, complications and patient's outcome at follow-up.

## MATERIALS AND METHODS

All patients who underwent endoscopic placement of SEMS or cSEMS for small bowel tumor obstruction (duodenum or jejunum) between August 2009 and September 2012 were retrospectively analyzed. Due to advanced or metastatic disease or comorbidity, none of the patients were considered candidates for curative surgical treatment of the tumor. All patients included into this study were symptomatic and were admitted to the hospital because of their obstructive symptoms including nausea, vomiting, bloating, or abdominal pain. At least for some time all patients were suffering from postprandial vomiting. No patient was treated for non-symptomatic stenosis. Indication for jejunal placement of stents was only given in cases of unilocular stenosis by circumscribed peritoneal

carcinomatosis. Histology was obtained by endoscopic biopsy from the intestinal tumor or percutaneous needle biopsy from liver metastasis. The therapeutic procedures were ascertained in an interdisciplinary conference with senior physicians from the departments of surgery, gastroenterology and medical oncology and the recommendation to treat by endoscopic stent placement was given in consent.

We retrospectively reviewed the prospectively collected records on technical success of the procedure, clinical benefit, and the incidence of complications including migration and stent occlusion. The patients' outcome at follow-up was additionally registered.

Patients were advised to resume oral intake of liquids within 24 h and to advance to a low-residue diet as tolerated. The status of oral food intake was monitored at one-month intervals on an outpatient basis. In case of recurrence of dysphagia, radiographic imaging (iodine or barium upper gastrointestinal series) and/or upper gastrointestinal endoscopy was performed. Patients who had recurrent symptoms caused by tumor overgrowth were treated by placement of a second intestinal stent.

### Endoscopic technique and stent selection

Stents were placed by very experienced gastroenterological endoscopists using a therapeutic gastroscope (GIF-1TQ 160), or a duodenoscope (TJF-Q180V, TJF-160 VR; all Olympus medical Europe, Hamburg, Germany) with a working channel of 3.7 or 4.2 mm, respectively. All stents were inserted through-the-scope (TTS) in combination with over-the-wire technique, and all stents were placed under fluoroscopic guidance. Selection of SEMS vs cSEMS was at the appraisal of the endoscopist and cSEMS was preferably chosen in case of advanced tumors with subtotal or complete obstruction intending to avoid later tumor ingrowth. SEMS was preferred over cSEMS in case that the investigator was in fear of blocking biliary outflow by crossing the duodenal papilla by the stent. If complications (migration or overgrowth) occurred, new stents were placed and the stenting procedure in the patient was switched from a covered to an uncovered stent and vice versa. The stents used in this study were self-expandable Nitinol uncovered SEMS (Niti-S, D-Type, TaeWoong medical, South Korea) and covered SEMS [cSEMS; Niti-S pyloric duodenal covered stent (End Bare Type), TaeWoong medical, South Korea]. A stent diameter from 18 to 28 mm and a stent length from 40 to 120 mm were used. The cSEMS provides a silicone covering and has a retrieval suture for preventing tumor-ingrowth and easy removal. It contains a fixed-cell braided structure. The SEMS is a fine mesh tubular prosthesis that facilitates immediate and continuous wall apposition due to the so-called D-weaving technology, *i.e.*, stent cells are unfixed resulting in a high flexibility and retaining its shape even in bending sections of the intestinal tract. In case that a stent did not cover the entire tumor obstruction, two overlapping stents were implanted to bridge the entire obstructed bowel segment and this was accounted

**Table 1 Patients' characteristics *n* (%)**

Characteristics	SEMS	cSEMS
Stents	16 (50)	16 (50)
Male gender	7 (44)	10 (63)
Age (yr), mean $\pm$ SD (range)	70 $\pm$ 11 (50-85)	71 $\pm$ 11 (50-84)
Localization		
Jejunum, <i>n</i>	3	0
Duodenum, <i>n</i>	13	16
Disease		
Pancreatic carcinoma, <i>n</i>	6	7
Cholangiocellular carcinoma, <i>n</i>	3	2
Gallbladder carcinoma, <i>n</i>	1	2
Gastric cancer, <i>n</i>	3	2
Colorectal cancer, <i>n</i>	2	0
Breast cancer metastasis, <i>n</i>	1	0
Stenosis due to duodenal ulcer perforation, <i>n</i>	0	3
Balloon dilatation of the stent	3 (19)	2 (13)
Concomitant biliary drainage	9 (56)	8 (50)

SEMS: Self-expanding metal stent; cSEMS: Covered SEMS.

as a single application in this study.

### Definition

Tumor overgrowth of the stent was defined as deterioration of the patient's condition (recurrence of dysphagia) and detection of narrowing of the stent lumen within or adjacent to the proximal or distal end of the stent mesh as a result of tumor growth, as shown by endoscopic and/or radiologic findings. Tumor ingrowth was defined as tumor obstruction through the stent mesh as a reason for as deterioration of the patient's condition (recurrence of dysphagia). Improvement of vomiting or the intake of fluids or food was assessed qualitatively. Postinterventional complication rate was defined as occurrence of complications within 24 h after stent placement, all other complications were observed until the end of the follow-up period.

### Ethics

The retrospective study was approved by the institutional review board (Ethikkommission) of the Johann Wolfgang Goethe-University Hospital.

### Statistical analysis

The present study is a retrospective cohort study. The primary endpoints were complications due to stent implantation including tumor overgrowth and stent migration. The secondary end points were effect of concomitant biliary stenting on migration rates and overall survival. Statistical analyses were performed with SPSS (Version 22.0, IBM, NY, United States). Predictors of survival were determined using a univariate Cox regression hazard model. Death was recorded as event. Survival curves with the estimated hazards were calculated with the Cox regression model. The patients at risk at the individual time points are shown in the figures. The data was analyzed according to the Strengthening the Reporting of Observational Studies in Epidemiology guidelines<sup>[14]</sup>.

**Table 2 Complications *n* (%)**

Complications	SEMS	cSEMS	<i>P</i> value
Duration of procedure (min), median (range)	60 (40-121)	60 (31-160)	0.867
Migration	0 (0)	9 (56)	0.002
Time until migration (d), mean $\pm$ SD (range)	-	30 $\pm$ 52 (1-161)	NA
Tumor overgrowth	3 (19)	2 (13)	0.725
Tumor ingrowth, <i>n</i>	0	0	NA
Time until tumor overgrowth (d), mean $\pm$ SD (range)	143 $\pm$ 95 (39-224)	96 $\pm$ 105 (22-170)	0.572
Overall survival (d), median, range	40 (3-275)	75 (11-426)	0.431

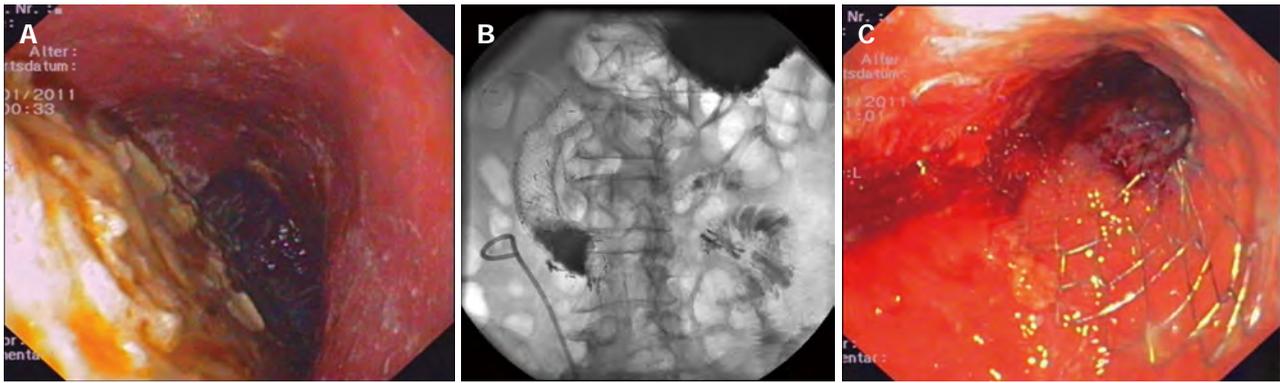
NA: Not available; SEMS: Self-expanding metal stent; cSEMS: Covered SEMS.

## RESULTS

Thirty-two cases of stent insertion were included in this study: 16 cSEMSs and 16 SEMSs were placed in 20 patients. Patient characteristics are shown in Table 1. Five patients received two overlapping stents; in the remaining 27 interventions a singular stent was put in place. Whereas all covered SEMS were implanted in the duodenum, 3 of 16 uncovered SEMS were inserted into the jejunum. The main etiology of gastric outlet obstruction was pancreatic cancer, followed by cholangiocellular carcinoma or gallbladder carcinoma (Table 1). All three jejunal SEMS were placed due to an obstruction that was caused by a circumscribed manifestation of peritoneal carcinomatosis in gastric cancer patients. Nine of the SEMS (56%) and eight of the cSEMS (50%) were placed in patients who presented with concomitant biliary tract stenosis; all these patients were treated with placement of plastic stents or SEMS/cSEMS into the common bile duct (CBD), and plastic endoprosthesis were replaced by biliary SEMS at the time of duodenal SEMS insertion in all patients.

We observed technical success of SEMS placement in all cases without occurrence of peri-interventional complications. In three (SEMS) and two (cSEMS) stent placements, respectively, balloon dilatation was needed for complete expansion of the stents. The duration of endoscopic procedure did not significantly differ between SEMS and cSEMS (Table 2). The clinical condition ameliorated in 14 of 16 (87.5%) cases treated with SEMS and intake of fluids or food improved. In contrast, in patients treated with cSEMS the clinical condition improved in 12 of 16 (75%) cases only. However, this difference was not statistically significant ( $P = 0.564$ ).

The mean follow-up time  $\pm$  SD was 57  $\pm$  71 d with a range of 1-275 d. In patients with gastric outlet obstruction and concomitant biliary obstruction no migration or occlusion of the bile duct stents was observed. Nine of the 16 cSEMS (56%) migrated within the observation time. In one of the patients the dislodged stent had to be recovered from the jejunum by laparotomy. The patient



**Figure 1** Self-expanding metal stents insertion in duodenal tumor obstruction. A: Retention of secretions and food in the stomach; B: Duodenal self-expanding metal stents (SEMS) together with biliary SEMS in X-ray; C: SEMS in endoscopic imaging.

was dismissed from hospital treatment after recovery from the surgery. On the contrary none of the SEMS dislocated. SEMS placement was superior to cSEMS for duodenal location of obstructions concerning migration rate (0% *vs* 56%). However, although none of the three SEMS placed in the jejunum migrated within time no further conclusion can be drawn for a superiority of SEMS in the jejunal location as no cSEMS was placed in the jejunum. The mean time until migration  $\pm$  SD was  $30 \pm 52$  d with a range of 1-161 d.

As stents in situ of the CBD might give hold to the duodenal SEMS/cSEMS and might be associated with a reduced rate of stent migration, we analyzed the group of patients with CBD SEMS/cSEMS and concomitant duodenal SEMS/cSEMS placement separately. In 17 of 32 cases, a combined biliary and duodenal SEMS/cSEMS insertion had been undertaken. Comparing the time until migration of stents in patients with and without biliary SEMS/cSEMS, there was no difference in migration of duodenal stents (HR = 1.530, 95%CI: 0.731-6.306;  $P = 0.556$ ). In those 17 patients in whom a biliary SEMS was in place, all six events of stent migration were observed in patients with duodenal cSEMS, whereas no migration was seen in patients with uncovered duodenal SEMS ( $P = 0.008$ ). A representative example of duodenal stent with concomitant biliary metal stent implantation is shown in Figure 1.

Overgrowth of a SEMS by progressive cancer occurred in three cases, whereas this complication was seen in two of the cSEMS patients. There was no tumor ingrowth into any of the stents (SEMS or cSEMS) observed. In case of tumor overgrowth or migration, a new stent was placed into the stenosis and migrated cSEMS were replaced by uncovered SEMS. A flow chart demonstrating the algorithm of stent treatment is displayed in Figure 2.

Thirteen patients died within the observation time. The median overall survival was 74 d (Figure 3A). To assess the influence of the stent type on survival, overall survival times for the two kinds of stents were compared. There was no significant difference between SEMS and cSEMS concerning overall survival according to a uni-

variate Cox regression model (Figure 3B).

## DISCUSSION

In this comparative study, we observed a high rate (> 50%) of stent migration in the covered SEMS group in comparison to the non-covered SEMS group (0%) in palliation of duodenal or small bowel obstruction. Concurrently, technical feasibility of stent placement (TTS technique) was similar, and relief of symptoms was equal in both patient groups. Insertion of SEMS was as safe as cSEMS implantation during peri-interventional surveillance of the patient. Thereby, biliary SEMS did not prevent migration of duodenal cSEMS migration, and the migration rate in patients with and without concomitant biliary stents was similar. We did not observe clinically significant tumor ingrowth in the SEMS or cSEMS group. However, the overall survival times were quite short and in patients with longer survival times cSEMS might show advantages concerning tumor ingrowth rates.

Randomized trials comparing SEMS *vs* cSEMS treatment in the small bowel has not been reported up to date, but an increased risk of stent migration in covered SEMS has been reported in colonic tumor palliation: Stent migration was four times as common in the covered SEMS group as in the non-covered stent group in a recent meta-analysis<sup>[15]</sup>. In patients with inoperable gastric cancer, comparison of cSEMS *vs* SEMS yielded similar results: Migration was observed in 26.0% of patients, in comparison to 2.8% in non-covered SEMS in a randomized trial<sup>[16]</sup>. Our results suggest that migration rate in the small bowel is even higher than in the stomach or large bowel. Technical success rate and clinical success was similar in cSEMS *vs* SEMS in both studies, and immediate complications were near to zero. Migration rates are low (1%-3% of cases) in other studies reporting on SEMS insertion in the duodenum and small bowel<sup>[1,17]</sup>.

As the duration of the endoscopic treatments and the responsible endoscopists did not differ between the two cohorts, we consider two factors to contribute mainly on migration in cSEMS *vs* SEMS study: First, the stent cover minimizes hyperplastic tissue to get hold of the stent

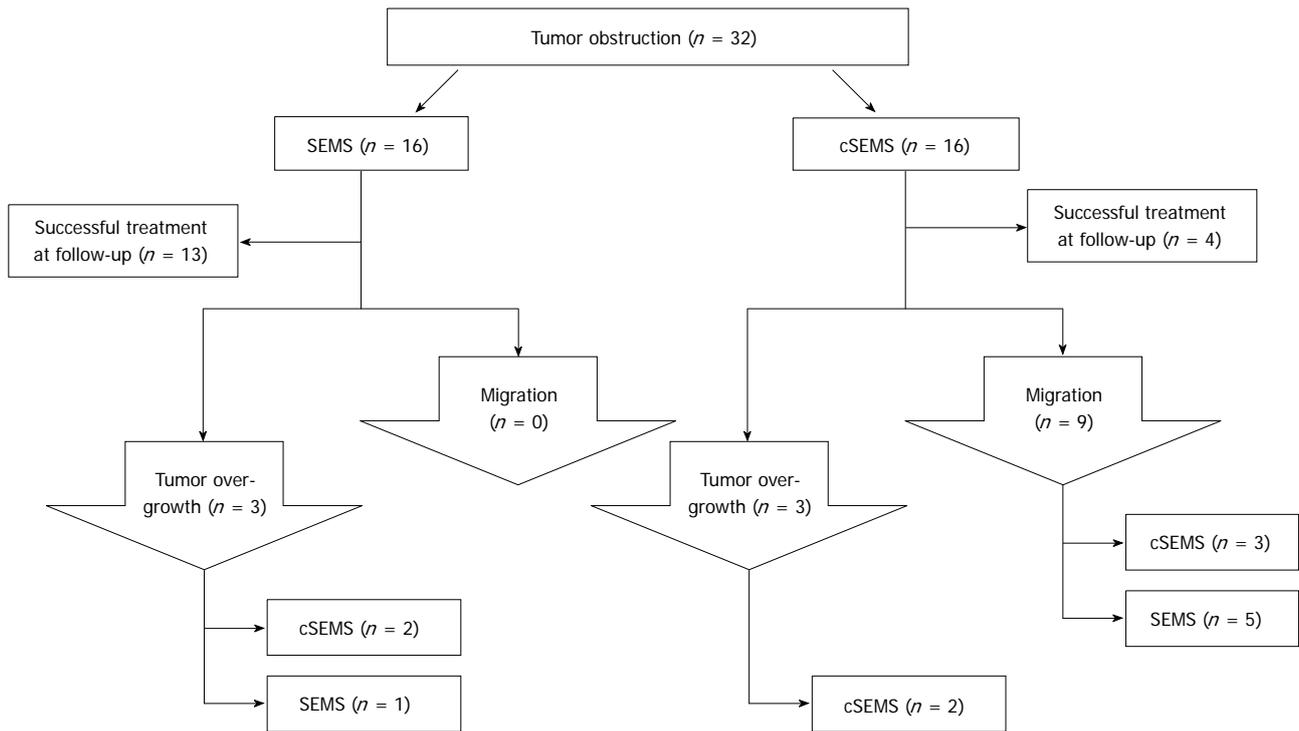
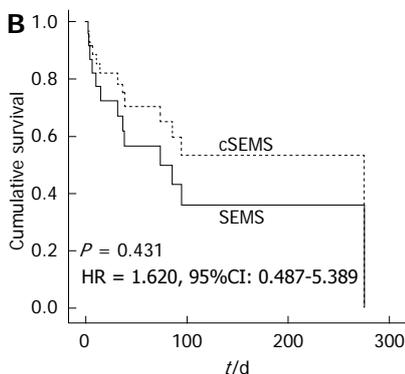
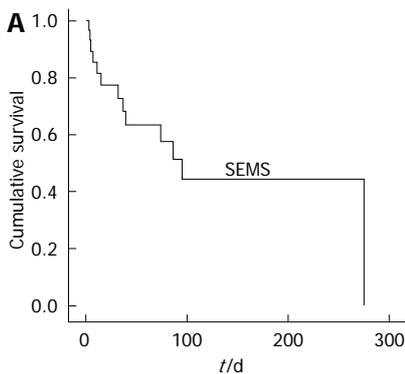


Figure 2 The treatment algorithm for self-expanding metal stents placement. SEMS: Self-expanding metal stents; cSEMS: Covered SEMS.



Cases at risk (cSEMS)	16	2	0
Cases at risk (SEMS)	16	4	2

Figure 3 Survival curves. A: The overall survival curve calculated with the Cox regression model; B: Survival curves for self-expanding metal stents (SEMS) and covered SEMS (cSEMS) placements calculated with the Cox regression model.

mesh and partial ingrowth of tumorous and hyperplastic tissue is prevented, thus minimizing any anti-migration effect that the stent may provide against the natural motility of the small bowel. Moreover, we think that the fixed-cell braided structure of the SEMS we used might have significantly contributed to migration by causing the stent to straighten itself by use of its axial expansion force. In opposite, the so-called D-weaving technology of the SEMS with unfixed stent cells might have resulted in a higher flexibility and retention of its shape even at bending sections of the intestinal tract, such as the duodenum. The motility of the small bowel contributes to the progression of the stent. Stent migration can be reduced by endoscopic clip fixation of stents in the duodenal position<sup>[18]</sup>. This procedure might be impracticable and only short-lasting, though, as clips usually dislodge in the short term.

The use of SEMS in duodenal obstruction might be compromised by concomitant biliary obstruction. However, combined endoscopic treatment is safe and successful in the majority of cases with only minor complications (0%-16% of cases)<sup>[19-23]</sup> even if others report found much higher incidence of complications (up to 52%)<sup>[24]</sup>. In our cohort of patients there were no treatment related complications of biliary drainage by duodenal stents. Migration rates were also not affected by the existence of biliary stenting. However, all events of migration of duodenal stents in patients receiving combined biliary and duodenal stenting happened in patients who had obtained cSEMS. Therefore, biliary stenting may protect from duodenal stent migration, if the biliary stents are placed in between the meshes of uncovered SEMS. The

**Table 3** Complication rates in small bowel self-expanding metal stents placement in retrospective analyses

Study	Patients, stents	Site of tumor obstruction	Tumor overgrowth	Migration	Bleeding	Perforation
Costamagna <i>et al</i> <sup>[11]</sup>	202, 212	Endoscopic duodenal stenting	12.4%	1.5%	3.0%	0.5%
van Hooft <i>et al</i> <sup>[21]</sup>	50, 57	Endoscopic stenting for gastric outlet obstruction	21.0%	4.0%	0.0%	0.0%
Jeong <i>et al</i> <sup>[41]</sup>	25, 28	Gastrojejunostomy, esophagojejunostomy, cSEMS	17.0%	4.0%	0.0%	1.0%
Chandrasegaram <i>et al</i> <sup>[7]</sup>	26, 31	Endoscopic stenting vs operative gastrojejunostomy	12.0%	0.0%	0.0%	0.0%
Jang <i>et al</i> <sup>[17]</sup>	583, 603	Peripyloric region, nonperipyloric region, duodenum alone anastomosis (Billroth I, Billroth II), jejunum	3.8%	NM	NM	< 1.0%
Kim <i>et al</i> <sup>[25]</sup>	50, 50	Endoscopic stenting for malignant gastroduodenal obstructions	18.0%	10.0%	0.0%	0.0%
Wong <i>et al</i> <sup>[26]</sup>	6, 6	Surgical vs endoscopic palliation	NM	NM	NM	NM
Mosler <i>et al</i> <sup>[27]</sup>	36, 52	Endoscopic stenting of nonesophageal upper gastrointestinal stenosis	11.0%	14.0%	0.0%	6.0%
Kim <i>et al</i> <sup>[28]</sup>	213, 236	Malignant gastroduodenal obstruction	7.0%	4.0%	1.0%	0.0%
Bang <i>et al</i> <sup>[29]</sup>	134, 132	Endoscopic treatment for malignant antropylic and duodenal	cSEMS 5.7% SEMS 19.0%	cSEMS 26.4% SEMS 6.3%	2.2%	< 1.0%
Keränen <i>et al</i> <sup>[30]</sup>	104, 130	Endoscopic treatment for malignant gastric outlet obstruction	18.0%	0.0%	0.0%	1.9%
Ahn <i>et al</i> <sup>[31]</sup>	47, 52	Malignant gastroduodenal obstruction, uncovered SEMS	11.0%	2.0%	0.0%	4.0%
Canena <i>et al</i> <sup>[32]</sup>	74, 80	Endoscopic stenting for gastric outlet obstruction	9.5%	0.0%	1.4%	0.0%
Cha <i>et al</i> <sup>[33]</sup>	85, 85	Endoscopic stenting for gastroduodenal obstruction	29.0%	4.0%	4.0%	4.0%
Own data	20, 32	Small bowel/duodenum	cSEMS 13.0% SEMS 19.0%	cSEMS 56.0% SEMS 0.0%	0.0%	0.0%

SEMS: Self-expanding metal stent; cSEMS: Covered SEMS; NM: Not mentioned.

advantage of SEMS in comparison to cSEMS might be lower rates of tumor occlusion<sup>[1,2,19]</sup>. In contrast in patients receiving cSEMS recurrence of tumor occlusion is rarely observed<sup>[19]</sup>. The rate of stent occlusion did not significantly differ between patients with SEMS (19%) and cSEMS (13%) in our study, though. Also in another report no differences were found between SEMS and SEMS concerning necessity of re-interventions<sup>[25]</sup>. An overview of literature concerning complications of SEMS placements is provided in Table 3.

The limitation of the current study is the retrospective non-randomized approach. However, in conclusion, technical feasibility, tumor overgrowth, and overall survival of the patients are comparable in SEMS vs cSEMS, but migration rate is much higher in cSEMS as migration was observed in none of the SEMS group patients.

We prefer SEMS over cSEMS insertion as first choice for malignant duodenal and small bowel obstruction and restrict use of cSEMS in cases with fast tumor ingrowth. Prospective randomized trials are needed to compare SEMS and cSEMS for small bowel obstruction.

comparing covered SEMS and uncovered SEMS in for tumor related stenosis has been reported. The authors hypothesized, that covered SEMS and uncovered SEMS differ concerning complications in the indicated localization.

### Innovations and breakthroughs

The authors learnt that covered SEMS showed significant higher migrations rates than uncovered SEMS when placed in the duodenal position. In contrast, no significant differences concerning re-obstruction of the lumen or overall survival after SEMS implantation were found.

### Applications

The authors conclude that uncovered SEMS should be preferred when SEMS implantation in the duodenum is performed.

### Terminology

SEMS are self-expanding metal stents which are often made of nitinol alloyings and are placed by guide wired and under fluoroscopic control in endoscopic procedures. SEMS can be used uncovered or covered with silicone or other materials.

### Peer review

The authors showed in their retrospective analysis for the first time that uncovered SEMS might be preferred for malignant duodenal or small stenosis, as covered SEMS show much higher rates of migration. The results may help to reduce complications raised by migrated SEMS.

## COMMENTS

### Background

Duodenal and small bowel obstructions are complications of advanced cancer disease. Endoscopic implantation of nitinol based self-expanding metal stents (SEMS) is a safe and established procedure for palliative treatment of tumor obstruction. Covered and uncovered SEMS differ in their characteristics concerning risk of tumor ingrowth and overgrowth or migration and dislocation. But up to now, there is no data provided that covered or uncovered SEMS should be favored for treatment of tumor stenosis in the duodenum or small bowel.

### Research frontiers

Endoscopic treatment of duodenal or small bowel obstruction is an established treatment procedure for malignant stenosis. However, no thorough analysis

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## Inflammatory bowel disease serology in Asia and the West

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### Abstract

**AIM:** To study serological antibodies in Caucasians and Asians, in health and inflammatory bowel disease (IBD), in Australia and Hong Kong (HK).

**METHODS:** Anti-glycan antibodies [anti-chitobioside (ACCA), anti-laminaribioside (ALCA)], and anti-mannobioside (AMCA), anti-*Saccharomyces cerevisiae* (gASCA); and atypical perinuclear anti-neutrophil cytoplasmic antibody (pANCA) were tested in IBD patients, their unaffected relatives, and healthy controls in Australia and HK (China). Antibody status (positive or negative) and titre was compared between subjects of different

geography, ethnicity and disease state.

**RESULTS:** Ninety subjects were evaluated: 21 Crohn's disease (CD), 32 ulcerative colitis (UC), 29 healthy controls, and 8 IBD patient relatives. Forty eight subjects were Australian (29 Caucasian and 19 ethnic Han Chinese) and 42 were from HK (all Han Chinese). Caucasian CD patients had a significantly higher antibody prevalence of gASCA (67% vs 3%,  $P < 0.001$ ), ALCA (44% vs 6%,  $P = 0.005$ ), and AMCA (67% vs 15%,  $P = 0.002$ ), whereas HK CD patients had a higher prevalence of only AMCA (58% vs 25%,  $P = 0.035$ ), when compared with UC and healthy subjects in both countries. Caucasian CD had significantly higher gASCA prevalence (67% vs 0%,  $P < 0.001$ ) and titre (median 59 vs 9,  $P = 0.002$ ) than HK CD patients. Prevalence and titres of ALCA, ACCA and AMCA did not differ between CD in the two countries. Presence of at least one antibody was higher in Caucasian than HK CD patients (100% vs 58%,  $P = 0.045$ ). pANCA did not differ between countries or ethnicity.

**CONCLUSION:** Serologic CD responses differ between HK Asian and Australian Caucasian patients. Different genetic, environmental or disease pathogenic factors may account for these differences.

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**Key words:** Crohn's disease; Ulcerative colitis; Serological antibodies; Asia; Ethnic; Anti-*Saccharomyces cerevisiae* antibodies; Anti-chitobioside antibodies; Anti-laminaribioside antibodies; Anti-mannobioside antibodies; Atypical perinuclear anti-neutrophil cytoplasmic antibodies

**Core tip:** Serological antibodies to enteric antigens are a hallmark of inflammatory bowel disease (IBD) and may carry pathogenic and prognostic significance. There is limited information about their role and prevalence in Asian patients. We evaluated anti-glycan antibodies (anti-chitobioside, anti-laminaribioside, and anti-mannobioside), anti-*Saccharomyces cerevisiae*; and atypical

perinuclear anti-neutrophil cytoplasmic antibody in IBD patients, their unaffected relatives, and healthy controls in Australia and Hong Kong (China). Serologic responses were found to differ between Asian and Caucasian patients. Different genetic, environmental or disease pathogenic factors may account for these differences.

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## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are related to a mucosal immune response to antigenic stimulation from the gut microbiota on a background of genetic susceptibility<sup>[1]</sup>. Serological antibodies for inflammatory bowel disease (IBD) have a role as diagnostic markers for IBD and assist in disease stratification<sup>[2]</sup>.

Glycans are carbohydrate surface components, which can be found on immune cells, erythrocytes, tissue matrices and microorganisms. They likely reflect the interaction between the immune system and glycosylated cell wall components of microbiota such as fungi, yeast, and bacteria<sup>[3]</sup>. Anti-*Saccharomyces cerevisiae* (gASCA) (IgA and IgG) antibodies are directed against the cell wall mannan of the yeast *Saccharomyces* that shares homology with intestinal bacteria<sup>[4]</sup>. gASCA (antibodies against covalently immobilized mannan)<sup>[5]</sup> have been found to be comparable to "conventional" ASCA<sup>[6]</sup>. Anti-laminaribioside carbohydrate IgG antibodies (ALCA), anti-chitobioside carbohydrate IgA antibodies (ACCA), anti-mannobioside carbohydrate IgG antibodies (AMCA) were first reported in 2006<sup>[5]</sup> and discovered using GlycoChip glycan array technology<sup>[7]</sup>. These antibodies may allow differentiation of IBD from health, define between IBD subtypes, and have been associated with a more complicated CD behaviour<sup>[2,5]</sup>. Atypical perinuclear anti-neutrophil cytoplasmic antibody (pANCA) is regarded as a marker of UC, as it has a higher prevalence in UC than in CD or healthy controls<sup>[8]</sup>.

Until two decades ago IBD was rare in Asia<sup>[9]</sup>, but recent population-based and referral centre cohorts<sup>[10,11]</sup> have shown a rising incidence and prevalence of IBD in Asia<sup>[12]</sup>. These temporal trends in disease incidence and prevalence may provide insights into possible etiologic factors, such as genetic *vs* environmental. As serologic antibodies may represent an interface between a patient's genetic make-up and their environment, we hypothesised that evaluation of serologic responses in areas of increasing incidence may provide an insight into these complex interactions. Most data on serological antibodies are derived from North American or European cohorts. There are no publications of the prevalence of the anti-glycan

antibodies in Asian cohorts, either in Asia or in Asians abroad.

This study aimed to provide an initial insight into the prevalence and magnitude of the anti-glycan antibodies, and pANCA in IBD, compared to control groups, in Han Chinese (referred to as Asian) and Caucasian subjects in Australia and in Han Chinese subjects in Hong Kong (China).

## MATERIALS AND METHODS

### Patient population

Serum samples were obtained from consented consecutive subjects, regardless of disease extent or duration, from IBD centres in Melbourne, Australia and Hong Kong (China).

IBD diagnosis and differentiation into UC and CD was made based on accepted clinical, endoscopic, histopathological, and radiological findings. Patient characteristics are shown in Table 1. The healthy subjects consisted of patients undergoing a colonoscopy for a family history of cancer or polyps, with a subsequent normal colonoscopy. Eight first degree relatives of IBD subjects (2 of UC, 6 of CD) who were undergoing a colonoscopy for cancer screening were also studied. Signed informed consent was obtained from all participants. The study was approved by the Ethics Committees of St Vincent's Public and Private Hospitals Melbourne, and The Chinese University of Hong Kong.

### Serological analysis

After blood was taken, serum was immediately separated by centrifugation and then frozen at -80 °C until use. All sera were processed anonymously.

The IBDX ELISA kit was used to detect gASCA IgG, ALCA IgG, ACCA IgA, AMCA IgG, following the manufacturer's recommendations (Glycominds Ltd, Lod, Israel). The cutoff values were those supplied by the manufacturer: 50, 90, 60, 100 EUs for gASCA IgG, ACCA, ALCA, and AMCA, respectively. pANCA was performed using indirect immunofluorescence on ethanol and formalin-fixed neutrophil substrate slides.

For the titre of immune response of the anti-glycan antibodies, quartile scores for each serologic antibody were calculated, as described previously<sup>[6,13,14]</sup>. For each patient each antibody titre was assigned to a quartile score of 1 (lowest), 2, 3, or 4 (highest). By adding individual quartile scores for each glycan antigen a semi-quantitative quartile sum score (QSS) (range 4-16), representing the cumulative quantitative immune response toward all four antigens for each patient, was obtained.

### Statistical analysis

Using the suggested cut-off values for each antibody, positive or negative status was determined for each subject. In addition, antibody titres were divided into four groups based on the quartiles (see description above). Discrete parameters were assessed as percentages and

**Table 1 Subject demographics and disease characteristics n (%)**

Country	Group	Ethnicity	Group No.	Age (yr) mean ± SD	Female	Never smoker	Family history of IBD	CD (severe behaviour)	CD (ileocolonic location)	UC proctitis
Australia (n = 48)	Crohn's	Caucasian	9	29 ± 12	4 (44)	3 (33)	0 (0)	8 (89)	7 (78)	-
		Caucasian	10	37 ± 11	5 (50)	7 (70)	1 (10)	-	-	1 (10)
	Healthy	Asian	10	45 ± 14	2 (20)	8 (80)	0 (0)	-	-	3 (10)
		Caucasian	10	46 ± 12	5 (50)	4 (40)	0 (0)	-	-	-
		Asian	9	51 ± 11	4 (44)	7 (78)	1 (11)	-	-	-
Hong Kong (n = 42)	Crohn's	Asian	12	38 ± 15	7 (58)	7 (58)	1 (8)	3 (25)	9 (75)	-
		Asian	12	43 ± 12	5 (42)	12 (100)	0 (0)	-	-	2 (17)
	Healthy	Asian	10	50 ± 5	6 (60)	7 (78)	0 (0)	-	-	-
		Asian	8	34 ± 9	3 (38)	6 (75)	8 (100)	-	-	-
	Total		90	42 ± 13	41 (46)	61 (68)	11 (12)	11 (52)	16 (76)	6 (19)

CD: Crohn's disease; UC: Ulcerative colitis; Severe behaviour: Strictureing or penetrating disease.

compared using Fisher's exact or  $\chi^2$  test where appropriate. Continuous parameters were assessed as means if normally distributed (compared using one way ANOVA), and medians if not normally distributed (compared using Mann-Whitney *U* test). The software Graphpad Prism 5 and SPSS 21 were used for analyses. *P* < 0.05 was considered statistically significant.

## RESULTS

### Demographics

Ninety participants (21 CD, 32 UC, 29 healthy controls, and 8 relatives of IBD patients) were divided according to geography, ethnicity and disease (Table 1). All Asian patients were Han Chinese. There was no significant difference when comparing age or gender distribution between countries (Australian *vs* HK subjects), or ethnicities (Asian *vs* Caucasian subjects).

### CD vs non-CD (UC, healthy subjects and relatives)

**Anti-glycan antibody prevalence and number of antibodies positive:** As the anti-glycan antibodies are known to be associated with CD, we compared each CD *vs* non-CD groups in combined Australian and HK cohorts. Three (gASCA, ALCA, AMCA) of the four anti-glycan antibodies were present in a significantly higher proportion of Australian Caucasian CD compared to all non-CD subjects combined [6/9 (67%) *vs* 2/69 (3%), *P* < 0.001; 4/9 (44%) *vs* 4/69 (6%), *P* = 0.005; and 6/9 (67%) *vs* 10/69 (15%), *P* = 0.002, respectively]. In contrast, in the HK Asian CD group only AMCA had a significantly higher proportion of subjects positive compared to all non-CD groups combined [7/12 (58%) *vs* 10/69 (15%), *P* = 0.002] (Table 2).

The proportion of subjects with at least one, and at least two, antibodies positive was significantly higher in the Australian Caucasian CD group than all non-CD groups combined [9/9 (100%) *vs* 17/69 (25%), *P* < 0.001; 6/9 (67%) *vs* 5/69 (7%), *P* = 0.001]. The HK Asian CD group had a significantly higher proportion of subjects with at least one antibody positive compared to all non-CD groups combined, [7/12 (58%) *vs* 17/69 (25%), *P* =

0.035], however, only 2/12 (17%) had at least two antibodies positive. All subjects in the HK Asian CD group that had an antibody positive had AMCA as one of the antibodies.

**Anti-glycan antibody titres:** The titres of three of the four anti-glycan antibodies (gASCA, ALCA, and AMCA), and the quartile sum score (QSS), were significantly higher in the Australian Caucasian CD group than all non-CD groups combined (median titres 59 *vs* 9, *P* < 0.001; 45 *vs* 18, *P* = 0.002; 111 *vs* 67, *P* = 0.002; 14 *vs* 9, *P* < 0.001, respectively). Two of the four anti-glycan antibodies (ALCA, and AMCA), and the QSS, had significantly higher titres in the HK Asian Crohn's group than all non-CD groups combined (median titres 27 *vs* 18, *P* = 0.029; 121 *vs* 67, *P* = 0.003, 13 *vs* 9, *P* = 0.022, respectively). HK relatives did not have a significantly higher number of antibodies positive, or a higher antibody titre, than other healthy subjects.

### CD in Australian Caucasians and Hong Kong Asians

**Anti-glycan antibody prevalence and number of antibodies positive:** The proportion of subjects with positive gASCA was significantly higher in the Australian Caucasian CD group than the HK Asian CD group [6/9 (67%) *vs* 0/12 (0%), *P* < 0.001]. Prevalence of ALCA, ACCA and AMCA in Australian Caucasian CD patients [4/9 (44%), 2/9 (22%), and 6/9 (67%)] was not significantly different to the HK CD patients [1/12 (8%), 1/12 (8%) and 7/12 (58%)]. The proportion of subjects with at least one antibody, or at least two antibodies, positive was significantly higher in Australian Caucasian CD patients than the HK Asian CD patients [9/9 (100%) *vs* 7/12 (58%), *P* = 0.045; 6/9 (67%) *vs* 2/12 (17%), *P* = 0.032].

**Anti-glycan antibody titres:** A significant difference was seen when comparing gASCA titres of Australian Caucasian CD to HK Asian CD patients (median titres 59 *vs* 9, *P* = 0.002). There was no significant difference in any other antibody titre, or the QSS, between the CD patients in the two countries.

**Table 2** Antibody positivity and titre according to geography, ethnicity and disease *n* (%)

	Australia					Hong Kong (all Asian)				
	CD		UC		Healthy		CD	UC	Healthy	Relatives
	Caucasian	Caucasian	Asian	Caucasian	Asian					
Total	9	10	10	10	9	12	12	10	8	
Antibody positivity										
gASCA	6 (67) <sup>a,c</sup>	0 (0)	1 (10)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
ALCA	4 (44) <sup>a</sup>	1 (10)	0 (0)	2 (20)	0 (0)	1 (8)	1 (8)	0 (0)	0 (0)	
ACCA	2 (22)	1 (10)	0 (0)	2 (20)	3 (33)	1 (8)	1 (8)	1 (10)	0 (0)	
AMCA	6 (67) <sup>a</sup>	0 (0)	0 (0)	1 (10)	3 (33)	7 (58) <sup>a</sup>	2 (17)	2 (20)	2 (25)	
pANCA	0 (0)	7 (70) <sup>e</sup>	5 (50) <sup>e</sup>	0 (0)	1 (11)	3 (25)	4 (33) <sup>e</sup>	0 (0)	0 (0)	
No. of positive antibodies										
At least 1	9 (100) <sup>a,c</sup>	2 (20)	1 (10)	2 (20)	4 (44)	7 (58) <sup>a</sup>	4 (33)	2 (20)	2 (25)	
At least 2	6 (67) <sup>a,c</sup>	0 (0)	0 (0)	2 (20)	2 (22)	2 (17)	0 (0)	1 (10)	0 (0)	
Antibody/QSS titre median (range)										
gASCA	59 (146) <sup>a,c</sup>	10 (12)	9 (47)	17 (45)	8 (21)	9 (44)	2 (39)	11 (17)	5 (46)	
ALCA	46 (79) <sup>a</sup>	17 (86)	14 (38)	23 (77)	18 (34)	27 (69) <sup>a</sup>	17 (82)	17 (18)	24 (25)	
ACCA	50 (188)	39 (101)	43 (56)	60 (310)	76 (135)	50 (80)	37 (87)	46 (77)	34 (62)	
AMCA	111 (154) <sup>a</sup>	63 (47)	70 (60)	79 (59)	75 (116)	121 (459) <sup>a</sup>	60 (272)	59 (145)	74 (94)	
QSS	14 (5) <sup>a</sup>	9 (6)	10 (6)	12 (8)	9 (10)	13 (10) <sup>a</sup>	8 (7)	8 (10)	10 (9)	

<sup>a</sup>Significantly higher than all non-Crohn's disease (CD) groups combined ( $P < 0.05$ ); <sup>b</sup>Significantly higher than Hong Kong Asian CD ( $P < 0.05$ ); <sup>c</sup>Significantly higher than all non-ulcerative colitis (UC) groups combined ( $P < 0.05$ ). gASCA: Anti-*Saccharomyces cerevisiae*; ALCA: Anti-laminaribioside carbohydrate IgG antibodies; ACCA: Anti-chitobioside carbohydrate IgA antibodies; AMCA: Anti-mannobioside carbohydrate IgG antibodies; pANCA: Atypical perinuclear anti-neutrophil cytoplasmic antibody; QSS: Quartile sum score.

### pANCA presence

The proportion of subjects with a positive pANCA in the Australian Caucasian UC group (7/10, 70%) did not differ significantly from the Australian Asian UC (5/10, 50%) and the HK Asian UC (4/12, 33%) patients. pANCA was present in 3/12 (25%) of the HK Asian CD group, but was virtually absent from all other non-UC groups. When comparing the Australian Caucasian UC patients, Australian Asian UC patients, and the HK Asian UC patients, to all non-UC subjects combined, each UC group had a statistically higher proportion of subjects with a positive pANCA ( $P < 0.001$ ,  $P = 0.002$ ,  $P = 0.025$ , respectively).

## DISCUSSION

There are very few studies reporting the prevalence of antibodies to microbial antigens in non-Western countries and between different ethnicities. This is the first report investigating anti-glycan antibodies in an Asian cohort, and the first report investigating pANCA in an Asian cohort residing in a country outside of Asia.

The prevalence of anti-glycan antibody in Australian Caucasian CD patients was consistent with previous published Western CD cohorts<sup>[14]</sup>, and were more prevalent than in all other subjects studied. The exception was ACCA which had a high prevalence in the healthy Australian Asian (33%) and Caucasian (20%) subjects, in contrast to a previously reported lower prevalence (0.5%-12%) in other healthy cohorts<sup>[2]</sup>.

gASCA was not present in any HK Asian CD subjects studied. This is in contrast to Asian data showing a similar prevalence of ASCA in Japanese<sup>[15]</sup> and South Korean<sup>[16,17]</sup> CD patients to that of Caucasian CD cohorts. A low gASCA titre was present in HK subjects. Chinese

patients in HK may not raise an antibody response to this antigen, or may do it only in low titre. Lawrence *et al*<sup>[18]</sup> directly compared a HK IBD cohort with an Australian Caucasian IBD cohort and found ASCA IgG detection was similar but IgA was lower in Chinese CD patients. This IgG detection may differ from the gASCA IgG we measured, although the two antibody measurements have been said to correlate well<sup>[6]</sup>.

Differences in prevalence of the anti-glycan antibodies may reflect true pathogenic differences in different populations. However they may still be present in some populations in low titre; this may need to be taken into account in non-Caucasian ethnicities.

AMCA was prevalent in Asian IBD patients and healthy Asian subjects. This antibody has low specificity for differentiating IBD from health in an Asian population. Bernstein *et al*<sup>[19]</sup> demonstrated a similar lack of specificity in a Canadian study of Caucasian and First Nations cohorts. He found a relatively high prevalence of IBD associated antibodies (pANCA, ASCA, anti-OmpC, anti-I2, and anti-CBir-1) in all First Nations cohorts (including controls). They concluded that these antibodies are unlikely to be of pathogenic significance.

pANCA was less prevalent in Asian UC than Caucasian UC patients. The lack of significance may relate to the small number of subjects studied and the modest difference observed. These findings are consistent with Asian UC studies from Japan (35%)<sup>[20]</sup>, South Korea (22%)<sup>[21]</sup>, and HK (44%)<sup>[18]</sup>. The prevalence in our Caucasian UC cohort was consistent with other Western UC cohorts<sup>[2]</sup>.

Our study included 8 first-degree relatives of IBD patients (all Asian from HK), six related to CD patients, and 2 to UC patients. The only 2 relatives with a positive anti-glycan antibody were related to a CD patient, and for both it was a positive AMCA. There have been no studies of

antibodies in relatives of IBD patients in Asian cohorts, however several studies have shown ASCA is present in 20%-56% of Caucasian healthy relatives of patients with CD<sup>[22-28]</sup>. None of the 8 relatives had a positive pANCA. Early studies of Caucasians demonstrated pANCA presence in 15%-30% of first degree relatives of patients with UC<sup>[29,30]</sup>, however this has not been replicated<sup>[31-36]</sup>, or not been significant when comparing to healthy non-related controls<sup>[37]</sup>.

This study has a number of limitations. Sample sizes were small; however these data provide a basis for larger confirmatory studies. Australian Caucasian CD patients had more severe disease than Hong Kong Asian CD patients which could be contributing to differences in antibodies<sup>[38]</sup>, however, because of the small numbers, comparisons between antibodies and CD phenotype were not made, but should be considered in further studies. Our lack of Australian Asian CD subjects limited our ability to separately determine the effects of ethnicity and geography. A cross sectional study on serological antibodies may be limited by changes in antibody status over time, although it appears that seropositive/seronegative antibody status remains relatively stable over time for the individual antibodies ASCA<sup>[13,14,23,38-41]</sup>, ALCA, ACCA and AMCA<sup>[14,38,42]</sup>.

In conclusion serological antibodies associated with IBD appear to differ in their presence and titre between the West and Chinese IBD patients. Caution should therefore be exercised in attributing pathogenic importance or using them as prognostic markers in different ethnic and geographic patient populations<sup>[43-45]</sup>.

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## COMMENTS

### Background

Serological antibodies to enteric antigens are a hallmark of inflammatory bowel disease (IBD) and may carry pathogenic and prognostic significance.

### Research frontiers

Until two decades ago IBD was rare in Asia, but recent population-based and referral centre cohorts have shown a rising incidence and prevalence of IBD in Asia.

### Innovations and breakthroughs

Although there has been previous research on serological antibodies in Caucasian patients with IBD, there is limited information about their role and prevalence in Asian patients in Asia, or in Asian migrants to the West.

### Applications

This study has found that serological antibodies associated with IBD appear to differ in their presence and titre between Western and Chinese IBD patients. Caution should therefore be exercised in attributing pathogenic importance or using them as prognostic markers in different ethnic and geographic patient populations.

## Terminology

Anti-*Saccharomyces cerevisiae* antibodies, which are directed against the cell wall mannan of the yeast *Saccharomyces*, that shares homology with intestinal bacteria; Antigliyan antibodies, which are directed against carbohydrates found on immune cells, erythrocytes, tissue matrices and microorganisms, and likely reflect the interaction between the immune system and glycosylated cell wall components of microbiota. The anti-glycan antibodies include: anti-chitobioside, anti-laminaribioside and anti-mannobioside; Perinuclear anti-neutrophil cytoplasmic antibody is widely regarded as a marker of ulcerative colitis.

## Peer review

This is interesting data of a little studied area in inflammatory bowel disease. The subject matter may be a spring board to further studies and understanding of the pathogenesis and prognosis of IBD.

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## Incidence and characteristics of HBV reactivation in hematological malignant patients in south Egypt

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### Abstract

**AIM:** To investigate characteristics of hepatitis B virus (HBV) implicated in HBV reactivation in patients with hematological malignancies receiving immunosuppressive therapy.

**METHODS:** Serum samples were collected from 53

patients with hematological malignancies negative for hepatitis B surface antigen (HBsAg) before the start of and throughout the chemotherapy course. HBV reactivation was diagnosed when the HBsAg status changed from negative to positive after the initiation of chemotherapy and/or when HBV DNA was detected by real-time detection polymerase chain reaction (RTD-PCR). For detecting the serological markers of HBV infection, HBsAg as well as antibodies to the core antigen (anti-HBc) and to the surface antigen were measured in the sera by CEIA. Nucleic acids were extracted from sera, and HBV DNA sequences spanning the S gene were amplified by RTD-PCR. The extracted DNA was further subjected to PCR to amplify the complete genome as well as the specific genomic sequences bearing the enhancer II/core promoter/pre-core/core regions (nt 1628-2364). Amplicons were sequenced directly.

**RESULTS:** Thirty-five (66%) of the 53 HBsAg-negative patients were found to be negative serologically for anti-HBc, and the remaining 18 (34%) patients were positive for anti-HBc. Five of the 53 (9.4%) patients with hematologic malignancies experienced HBV reactivation. Genotype D1 was detected in all five patients. Four types of mutant strains were detected in the S gene product of HBV strains and were isolated from 3 patients with HBV reactivation: T/S120, L143, and I126. HBV DNA was detected in the pretreatment HBsAg-negative samples in one of the five patients with HBV reactivation. In this patient, sequences encompassing the HBV full genome obtained from sera before the start of chemotherapy and at the time of *de novo* HBV hepatitis were detected and it showed 100% homology. Furthermore, in the phylogenetic tree, the sequences were clustered together, thereby indicating that this patient developed reactivation from an occult HBV infection.

**CONCLUSION:** Past infection with HBV is a risk factor for HBV reactivation in Egypt. Mandatory anti-HBc

screening prior to chemotherapy in patients with hematological malignancies is recommended.

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**Key words:** Hepatitis B virus; Occult infection; Reactivation; Hepatitis B surface antigen

**Core tip:** The study aimed to investigate characteristics of hepatitis B virus (HBV) implicated in HBV reactivation in patients with hematological malignancies receiving immunosuppressive therapy in Egypt. Fifty-three hepatitis B surface antigen (HBsAg)-negative patients treated with chemotherapy were included in the study. The incidence of HBV reactivation was 9.4% among the studied cohort, and all of the affected individuals were positive for HBsAg as well as antibodies to the hepatitis B core antigen. The present study provides further evidence via molecular evolutionary analysis of the development of HBV reactivation from an occult HBV infection. Past infection with HBV is a risk factor for HBV reactivation in Egypt. Mandatory antibodies to the core antigen screening prior to chemotherapy in patients with hematological malignancies is suggested.

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## INTRODUCTION

Infection with hepatitis B remains one of the major causes of acute and chronic liver disease. An estimated 350-400 million people are chronically infected with hepatitis B virus (HBV) worldwide<sup>[1]</sup>.

The reactivation of hepatitis B infection has been recorded in many clinical settings: chronic HBV infection after the cessation of HBV treatment, patients with malignant disease who receive immunosuppressant or chemotherapy, patients with end stage renal failure, and patients co-infected with human immunodeficiency virus (HIV)<sup>[2-6]</sup>. Patients with resolved HBV infection are diagnosed serologically by clearance of serum hepatitis B surface antigen (HBsAg) and the appearance of the hepatitis B core antibody (anti-HBc), with or without antibodies to hepatitis B surface antigen (anti-HBs)<sup>[7]</sup>. These patients are at risk of hepatitis B reactivation due to any factor that can suppress the immune system<sup>[8,9]</sup>. *De novo* hepatitis B is of particular concern in this subset of patients because it commonly leads to severe liver dysfunction and fatal hepatitis<sup>[10,11]</sup>.

Occult hepatitis B is defined by the presence of HBV DNA in the serum or the liver in the absence of HBsAg,

with or without anti-HBc or anti-HBs. In these patients, a low level of HBV replication has been shown to persist in the liver and in peripheral blood mononuclear cells for decades<sup>[12]</sup>. Occult HBV infection is observed worldwide, and its prevalence is related closely to the endemicity of HBV infection.

Large scale geographic heterogeneity in the prevalence of HBV had been reported worldwide. Africa is one of the highly endemic regions of HBV, and an intermediate endemicity of HBV infection had been recorded in Egypt<sup>[13,14]</sup>.

The aim of this study was to investigate the incidence of HBV reactivation and the underlying risk factors of hepatitis B reactivation in Egyptian patients who received cytotoxic chemotherapy for hematological malignancies.

## MATERIALS AND METHODS

### Patients

Fifty-nine consecutive patients with hematological malignancies were admitted to the oncology department of Sohag Faculty of Medicine and South Egypt Cancer Institution from November 2010 to October 2011. After admission, all patients underwent physical examination and blood and serum biochemistry analyses. All of patients received chest computed tomography and ultrasonography of the abdomen as an initial evaluation.

In clinical practice, patients are monitored during chemotherapy using liver function tests. HBsAg and HBV DNA are tested in patients with elevated liver enzymes. For the purpose of this study, serum samples were collected before and after the start of the chemotherapy course. The collected sera were stored at -80 °C for future examination of HBsAg, anti-HBs, and anti-HBc. HBV reactivation was diagnosed when the HBsAg status changed from negative to positive after the initiation of chemotherapy and/or when HBV DNA was detected as measured by real-time detection polymerase chain reaction (RTD-PCR) using stored samples from patients, as described latter.

### Serological markers of HBV infection

HBsAg was measured by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBc of the IgG class was determined by radioimmunoassay (Abbott Japan). All serologic assays were performed according to the manufacturer's instructions.

### Detection and quantitation of serum HBV DNA

HBV-DNA sequences spanning the S gene were amplified by RTD-PCR according to the previously described protocol with a slight modification and a detection limit of 100 copies/mL (equivalent to 20 IU/mL)<sup>[15]</sup>.

### Sequencing and molecular evolutionary analysis of HBV

Nucleic acids were extracted from serum samples (200 µL) using the QIAamp DNA extraction kit (Qiagen, Hilden,

**Table 1 Characteristics of 53 patients with malignant hematologic disease who were negative for hepatitis B surface antigen *n* (%)**

Characteristics	Total ( <i>n</i> = 53)	Anti-HBc positive ( <i>n</i> = 18)	Anti-HBc negative ( <i>n</i> = 35)	<i>P</i> value
Age yr, mean ± SD	27.8 ± 26.2	34.4 ± 27.9	27.7 ± 25.4	0.42
Gender (male)	26 (49.1)	10 (55.6)	16 (45.7)	0.56
Diagnosis				
Malignant lymphoma	26 (40.1)	9 (50.0)	17 (48.6)	1.00
Acute leukemia	25 (47.2)	9 (50.0)	15 (42.9)	0.77
Chronic leukemia	1 (1.9)	0 (0.0)	1 (2.9)	1.00
Multiple myeloma	1 (1.9)	0 (0.0)	1 (2.9)	1.00

Anti-HBc: Antibody to hepatitis B core antigen.

Germany).

Extracted DNA was subjected to PCR for amplifying the complete genome and the specific genomic sequences bearing enhancer II /core promoter/pre-core/core regions (nt 1628-2364), as described previously<sup>[16]</sup>.

Amplicons were sequenced directly using the ABI Prism Big Dye ver. 3.1 kit in the AMI 3100 DNA automated sequencer (Applied Biosystems; Foster City, CA, United States).

All sequences were analyzed in both the forward and reverse directions. HBV genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTALX, and genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/>)<sup>[17]</sup>. Based on the obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times for analysis by the ODN program of the National Institute of Genetics.

**Ethical consideration**

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from all patients.

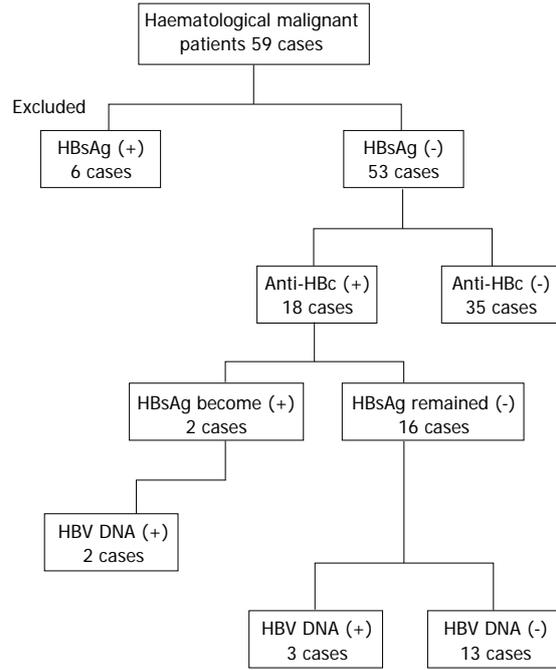
**Statistical analysis**

Statistical analysis was performed with the Fisher’s exact probability test and the independent *t* test for the continuous variables using the SPSS software package (SPSS, Chicago, IL, United States). *P* values (two-tailed) less than 0.05 were considered statistically significant.

**RESULTS**

**Patient characteristics**

Six of the 59 patients with hematologic malignancies were found to be HBsAg positive and were excluded from the analysis. Therefore, a total of 53 HBsAg-nega-



**Figure 1 Longitudinal representation of hepatitis B reactivation after chemotherapy in patients with hematological malignancies.** HBsAg: Hepatitis B surface antigen; anti-HBc: Antibody to hepatitis B core antigen; HBV: Hepatitis B virus.

tive patients were checked for the serological markers of infection with hepatitis B. The background general characteristics of the 53 HBsAg-negative patients are presented in Table 1. The mean age of the analyzed cohort was 27.8 ± 26.2 years old. Thirty-five (66%) of 53 HBsAg-negative patients were found to be anti-HBc-negative, and 18 (34%) patients were serologically positive for anti-HBc. The predominance of male patients was observed in both the anti-HBc-positive and -negative patient groups. Twenty-six patients (40.1%) were diagnosed with malignant lymphoma, whereas 25 patients (47.2%) were diagnosed with acute leukemia. Solitary cases of chronic leukemia and multiple myeloma were also included in the studied cohort. An insignificantly higher incidence of acute leukemia cases was observed in the anti-HBc-positive patients (9/18; 50%) compared with the anti-HBc-negative patients (15/35; 42.9%).

**Consequences of HBV serology after receiving anti-cancer treatment**

After the initiation of systemic chemotherapy, examination of the HBV serology revealed that two (3.8%) of the HBsAg-negative patients became serologically positive for HBsAg. In addition, 3 more patients (5.8%) exhibited detectable HBV DNA in their sera after the start of the anticancer therapy (Figure 1). Interestingly, none of the serologically negative patients for anti-HBc became serologically positive for HBsAg or molecularly detectable for HBV DNA. In contrast, 2 of the 18 anti-HBc-positive patients (11.1%) became serologically positive for the HBsAg, and 3 (16.7%) became molecularly detectable for the HBV DNA. In brief, 5 of the 53 HBsAg negative

**Table 2** Clinical and virological characteristics of patients who experienced hepatitis B reactivation

Characteristics	Case 1	Case 2	Case 3	Case 4	Case 5
Age (yr)/gender	79/F	8/M	11/F	5/M	20/M
Diagnosis	NHL (stage III)	AML	ALL	ALL	ALL
Treatment <sup>1</sup>	CVP	St Jude protocol	St Jude protocol	St Jude protocol	St Jude protocol
HBV serology and DNA prior to chemotherapy					
HBsAg/anti-HBs/HBV DNA (log copy/mL)	(-)/(+)/1.8	(-)/(+)/negative	(-)/(-)/negative	(-)/(+)/negative	(-)/(nt)/negative
HBV reactivation months after anti-cancer therapy	12	4	5	6	4
HBV serology and DNA after chemotherapy					
HBsAg/anti-HBs/HBV DNA (log copy/mL)	(+)/(nt)/7.6	(+)/(+)/5.8	(-)/(-)/3.1	(-)/(+)/2.9	(-)/(nt)/2.0
ALT (IU/mL)	35	195	27	86	17
Total bilirubin (mg/dL)	1	1.1	1.3	1.1	0.2
Outcome	Died	Died	Died	Alive	Alive
HBV genotype	D1	D1	D1	D1	D1
Core promoter mutation	Wild	T1764/G1766	A1764	Wild	-
Pre-core A1896	Mutant	Wild	Wild	Wild	-
Amino acid mutation in S gene product	P120S/S143L	P120T	-	T126I	-

M: Male; F: Female; NHL: Non-Hodgkin lymphoma; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; HBsAg: Hepatitis B surface antigen; Anti-HBs: Antibody to hepatitis B surface antigen; CVP: Cyclophosphamide, vincristine, prednisone; ALT: Alanine amino transferase enzyme. <sup>1</sup>St Jude protocol: (1) prephase: vincristine + steroid; (2) induction: vincristine + fludarabine + aracytine + etoposide, intrathecal; (3) consolidation: high dose methotrexate + mercaptopurine; (4) continuation: methotrexate + mercaptopurine.

patients (9.4%), representing 27.8% (5/18) of the anti-HBc-positive patients in the studied cohort, manifested the criteria of HBV reactivation (Figure 1).

### Clinical and virological criteria of the patients who manifested HBV reactivation

Five of the 53 patients (9.4%) treated for hematologic malignancies manifested HBV reactivation throughout the anti-cancer therapy regimen. The demographic, clinical and virological criteria of the HBV infection of the five patients who experienced HBV reactivation are summarized in Table 2 (cases 1-5). The mean age of the five patients was  $24.6 \pm 30.9$  years old. Three of the patients were males (cases 2, 4 and 5), and two were females. Four patients were diagnosed with acute leukemia (cases 2-5), and only one patient (case 1) was diagnosed with malignant lymphoma. All of the 5 patients received a steroid regimen as a part of their anticancer therapy. All 5 patients were positive for anti-HBc. Three patients were positive for anti-HBs (cases 1, 2 and 4), and only one patient was serologically negative for the anti-HBs (case 3). Because of small volume of serum sample obtained from case 5, anti-HBs could not be tested. After HBV reactivation, two cases (cases 2 and 4) exhibited abnormal ALT levels, and one patient (case 2) experienced a more than 3-fold increase in the ALT level, indicating the emergence of hepatitis in this patient. None of the 5 cases who experienced had the HBV reactivation after cancer chemotherapy received an antiviral treatment for HBV.

The virological and molecular criteria are summarized in Table 2. The infecting genotype of the HBV strains was HBV genotype D, subtype D1 in all five cases. Two core promoter HBV variants were detected in 2 patients.

The two variants were T1764/G1766 and A1764 in cases 2 and 3, respectively. The stop codon pre-core HBV mutant (A1896) was detected in one patient (case 1).

Infection with HBV mutant strains in the S gene product was detected in 3 patients. The amino acid escape mutant strains are as follows: S120 and L143 (case 1), T120 (case 2) and I126 (case 4). Four types of mutant strains (T/S120, L143, and I126) were detected in the S gene strains of 3 patients (cases 1, 2 and 4, respectively).

### DNA sequencing and phylogenetic analysis

HBV DNA was quantified retrospectively by RTD-PCR in the stored samples of the five patients with HBV reactivation. Evidence of occult HBV infection at the time of the HBsAg-negative status (before the start of anti-cancer therapy) was detected by RTD-PCR in one patient (case 1). To determine the source of HBV infection, sera from case 1 before (case 1-A) and at the time of HBV reactivation (case 1-B) were subjected to HBV full genome amplification and sequencing. Sequences encompassing the HBV full genome obtained from sera before the start of chemotherapy and at the time of *de novo* HBV hepatitis revealed 100% homology, and the two sequences clustered together in the phylogenetic tree (Figure 2). These results demonstrate that case 1 developed reactivation from an occult HBV infection.

## DISCUSSION

This study is considered the first step in documenting and characterizing the reactivation of hepatitis B in Egypt among patients negative for the HBsAg who received immunosuppressive therapy. The current study presented

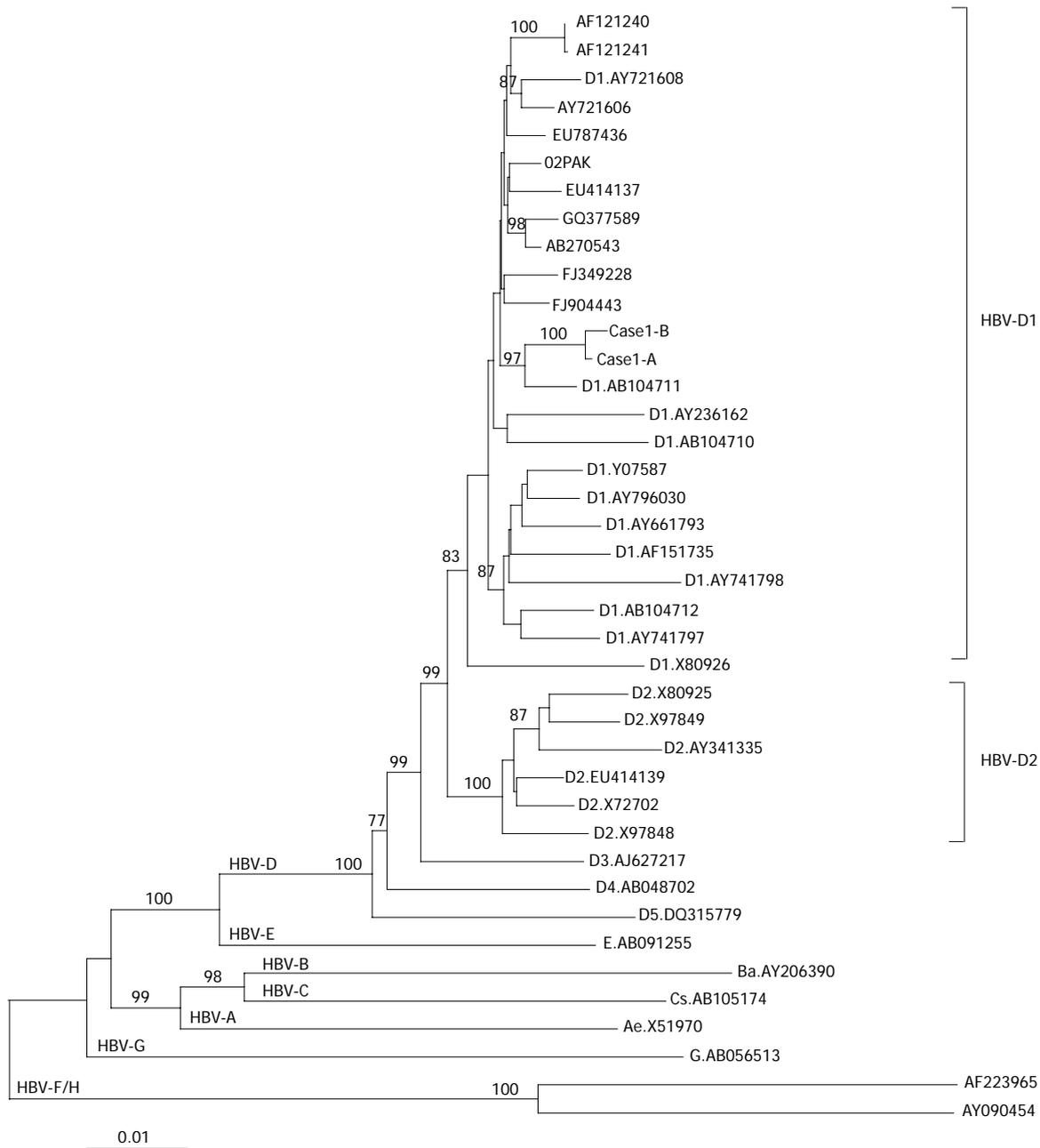


Figure 2 The complete genome of the hepatitis B virus was isolated and sequenced (case 1) prior to the start of chemotherapy (case 1-A) and after the emergence of hepatitis B virus reactivation (case 1-B). The phylogenetic analysis demonstrated that the patient (case 1) developed an hepatitis B virus (HBV) reactivation of an occult HBV infection.

further evidence that resolved hepatitis B infection and occult HBV infection may represent a hidden risk factor for the development of *de novo* hepatitis B.

The incidence of hepatitis B reactivation in the HBsAg-negative group was 9.4%, and all cases of reactivation occurred in patients with resolved or past infection with hepatitis B, as evidenced by the absence of HBsAg and the serological detection of anti-HBc. The patients who had HBV reactivation represent 27% of the HBsAg-negative/anti-HBc-positive patients. This incidence was comparable to the incidence that was described by Hui *et al.*<sup>[18]</sup>. In their study, Hui *et al.*<sup>[18]</sup> described an HBV reactivation incidence of 3.3% (8/244) in their studied cohort,

which included HBsAg-negative lymphoma patients receiving systemic chemotherapy. Of note, all 8 patients were seropositive for either anti-HBc or anti-HBs antibody. Recently, Matsue *et al.*<sup>[19]</sup> conducted a retrospective study on consecutive patients with CD20-positive B cell lymphoma before and after rituximab-containing treatment. In the latter study, 5 out of 230 patients negative for HBsAg (2.2%) experienced HBV reactivation, representing an incidence of 8.9% of the anti-HBc-positive patients<sup>[19]</sup>. In a prospective observational study of patients with hematological malignancies (a study cohort similar to the current study), Francisci *et al.*<sup>[20]</sup> reported the incidence of HBV reactivation was (18%), which is close

to that detected in the present study. The reasons for the difference in the incidence in HBV reactivation among different studies remain to be elucidated. However, the intensity of treatment, patient characteristics, and geographic differences in HBV prevalence and its genotypes may account for these differences<sup>[21]</sup>. Furthermore, the lack of a clear definition of HBV reactivation should not be ignored as a possible explanation for this variation in the incidence. In this study, the inclusion of patients who had detectable HBV DNA after cancer chemotherapy plus patients who exhibited HBsAg seroconversion after receiving the anticancer therapy dramatically increased the incidence of HBV reactivation among the studied cohort. This criterion of including cases with detectable HBV DNA after cancer chemotherapy as a sign of HBV reactivation was not used to define cases with HBV reactivation in the related studies<sup>[18,19]</sup>. The variations in the cohort size among the different studies cannot be ignored as a possible factor that may be implicated in such discrepancy.

Occult HBV infection is defined by the detection of HBV DNA in the sera or in the livers of serologically HBsAg-negative patients<sup>[14]</sup>. Until recently, the clinical effects of occult HBV infection were unclear regarding the influence on the progression of liver disease, the development of hepatocellular carcinoma, the risk for HBV reactivation, and the transmission of HBV infection<sup>[22]</sup>. The underlying mechanisms for the pathogenesis of occult HBV infection may be due to either viral or host factors<sup>[23]</sup>. One of the important viral factors is the presence of mutations in the HBV DNA sequence, which may interfere with the detection of HBsAg by the commercial assays, *i.e.*, “escape mutations”<sup>[24]</sup>. In the present study, 4 types of possible escape mutants were detected in 3 of the 5 patients who experienced HBV reactivation<sup>[25]</sup>. Previous *in vitro* studies have reported that escape mutations are associated with an increased immune evasive capacity and are capable of causing symptomatic flare up and high viral loads<sup>[26]</sup>. Furthermore, studying the viral genome isolated from case 1 revealed a complete match of the sequences obtained before the start of chemotherapy and at the time of reactivation. The present study provides further evidence of the emergence of HBV reactivation of occult hepatitis B as confirmed by the molecular evolutionary analysis<sup>[27]</sup>. Furthermore, two amino acid escape mutations in the S gene product, P120S and S143L, were detected in the HBV viral genome isolated from case 1.

Patients with malignancies in Egypt are monitored only by testing ALT levels throughout the chemotherapy course. Therefore, the present study, which is the first to explore HBV reactivation in Egypt, suggests mandatory serological screening for anti-HBc and anti-HBs in patients planning to receive immunosuppressant therapy. Patients found to be positive for anti-HBc, particularly patients who are negative for anti-HBs, should be closely monitored with HBsAg, HBV DNA and serum biochemistry during chemotherapy and for at least 6 mo after the completion of therapy. Further prospective multicenter studies are needed to explore the incidence

and risk factors of HBV reactivation in Egypt. Further studies are recommended to determine whether specific genomic mutations are implicated in *de novo* hepatitis in this subset of patients infected with HBV genotype D1.

## COMMENTS

### Background

The reactivation of hepatitis B is a syndrome characterized by an abrupt appearance or rise of the hepatitis B virus (HBV) DNA in the sera of patients with resolved or inactive hepatitis B infection. Reactivation can be spontaneous but is typically triggered by cancer chemotherapy, immune suppression or alterations in immune system function. Hepatitis B reactivation is of special clinical concern in immunocompromised patients because it leads to severe liver dysfunction and hepatic failure. However, hepatitis B reactivation is easy to prevent by introducing a prophylactic oral antiviral therapy. Occult hepatitis B is defined by the presence of HBV DNA in the serum or the liver in the absence of Hepatitis B surface antigen (HBsAg) with or without hepatitis B core antibody (anti-HBc) or antibodies to HBV surface antigen (anti-HBs). These patients are at risk of developing hepatitis B reactivation due to any factor suppressing the immune system. In Egypt, patients receiving cancer chemotherapy are typically monitored by liver function tests, with no screening for HBsAg or HBV DNA except in cases with elevated liver enzymes. This study aimed to investigate the incidence of HBV reactivation and the underlying risk factors of reactivation in Egyptian patients with hematological malignancies who were receiving cancer chemotherapy.

### Research frontiers

In a cohort of 53 patients with hematological malignancies receiving cancer chemotherapy who were negative for HBsAg, 18 patients (34%) were found to be positive for the anti-HBc, and five of the 53 (9.4%) patients with hematologic malignancies experienced HBV reactivation. All five patients were positive for anti-HBc. HBV DNA was detected in pretreatment HBsAg-negative samples in one of the five patients with HBV reactivation. In this patient, sera were obtained before the start of chemotherapy and at the time of *de novo* HBV hepatitis; the molecular evolutionary analysis of the sequences encompassing the HBV full genome obtained from the sera revealed that this patient developed reactivation from an occult HBV infection.

### Innovations and breakthroughs

This study is the first in Egypt to characterize HBV reactivation in Egypt. The study introduces more evidence through molecular evolutionary analysis that occult HBV infection is a risk factor for reactivation of hepatitis B in patients with hematological malignancies receiving cancer chemotherapy.

### Applications

The study strongly recommends mandatory serological screening for anti-HBc and anti-HBs in this subset of patients before the commencement of chemotherapy. Patients found to be positive for anti-HBc, particularly patients who are negative for anti-HBs, should be closely observed for signs of HBV reactivation through the regular monitoring of HBsAg and HBV DNA.

### Peer review

In the study, performance of sequencing and molecular analysis of HBV genomes seems relevant in characterization of the strains associated with HBV reactivation. Their findings are significant and beneficial for the readers.

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## Grasper type scissors for endoscopic submucosal dissection of gastric epithelial neoplasia

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### Abstract

**AIM:** To evaluate the efficacy and safety of grasper type scissors (GTS) for endoscopic submucosal dissection (ESD) of gastric epithelial neoplasia.

**METHODS:** The study was performed by 4 endoscopists in 4 institutions affiliated to The Catholic University of Korea. ESD was performed in 76 consecutive patients with gastric epithelial neoplasia by using the GTS (37 patients) or the hook knife plus coagrasper (HKC) (39 patients). The complete resection rate, complication rate, total time elapsed and elapsed time per

square centimeter of the dissected specimen were analyzed between the GTS and HKC group.

**RESULTS:** The mean age of the GTS group was  $62.3 \pm 11.4$  years and mean age of the HKC group was  $65.6 \pm 10.1$  years. Differentiated adenocarcinoma was found in 32.4% in the GTS group and 33.3% in the HKC group. The procedures were performed without interruption in every case in both groups. The *en bloc* resection rates of both groups were 100%. The total time elapsed during the procedure was  $44.54 \pm 21.72$  min in the GTS group and  $43.77 \pm 21.84$  min in the HKC group ( $P = 0.88$ ) and the time elapsed per square centimeter of the resected lesion was  $7.53 \pm 6.35$  min/cm<sup>2</sup> in the GTS group and  $6.92 \pm 5.93$  min/cm<sup>2</sup> in the HKC group ( $P = 0.66$ ). The overall complication rate was not significantly different between the two groups.

**CONCLUSION:** GTS is a safe and effective device for ESD compared with HKC. ESD can be performed with GTS alone, which can reduce the costs for ESD.

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**Key words:** Gastric epithelial neoplasia; Endoscopic submucosal dissection; Grasper type scissors; Hook knife; Coagrasper

**Core tip:** Many types of knives have been developed and used for endoscopic submucosal dissection (ESD). We modified the grasping type scissors forceps and developed a novel grasper type scissors (GTS). The aim of this study was to evaluate the efficacy and safety of GTS compared to hook knife plus coagrasper (HKC) for ESD of gastric epithelial neoplasia. The procedures were performed without interruption in every case in both groups. GTS is a safe and effective device for ESD of gastric epithelial neoplasia compared with HKC. ESD can be performed with GTS alone, which can reduce the costs for ESD.

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## INTRODUCTION

Endoscopic mucosal resection (EMR) is an endoscopic technique developed for removal of sessile or flat neoplasms confined to the superficial layers (mucosa and submucosa) of the gastrointestinal tract. EMR is typically used for removal of lesions smaller than 20 mm. *En bloc* resection of lesions larger than 20 mm is difficult by EMR thus increasing the risk of local recurrence<sup>[1-4]</sup>. Newly developed endoscopic techniques and devices have helped to overcome the limitations of EMR in terms of lesion size, location, and presence of fibrotic scarring.

Endoscopic submucosal dissection (ESD) allows *en bloc* resection of larger (usually more than 20 mm) lesions as well as subepithelial gastrointestinal lesions<sup>[5]</sup>. Various cutting devices - insulated tipped (IT) knife, hook knife, flex knife, triangular knife, and fork knife and so on-have been developed. Grasping-type scissors forceps (GSF) have a 0.8-mm-wide and 6-mm-long serrated cutting edge to facilitate the grasping of tissues. The outer side of the forceps is insulated and the forceps are able to rotate to the desired location. GSF can be used for excision by accurately gripping the submucosal tissue of the target lesion<sup>[6-8]</sup>. This device has been used safely and effectively for ESD in other organs such as the colorectum and duodenum<sup>[9-12]</sup>. However, it was not designed for cutting tissues and sometimes it was not optimal when used for dissecting lesions. Furthermore, rotating the GSF to the desired location is frequently difficult.

The newly developed grasper type scissors (GTS) can grasp and cut a piece of tissue using an electrosurgical current. Unlike GSF, the tip of the knife is not insulated and has a thin cutting blade to facilitate submucosal dissection. Theoretically, it possesses both advantages of the GSF and the flex knife.

The aim of this study was to evaluate the efficacy and safety of the novel GTS knife, which can be used for both dissection and hemostasis, for ESD of gastric epithelial neoplasia and compare it to hook knife plus coagrasper (HKC), which is one of the most commonly used knives in South Korea.

## MATERIALS AND METHODS

### Ethical consideration

The advantages and disadvantages of ESD with GTS, as well as alternative endoscopic options (*e.g.*, ESD with a conventional device, EMR) were discussed with each pa-

tients. All patients gave their written informed consent to the designated intervention. This study protocol was approved by the Institutional Review Board of The Catholic University of Korea.

### Patients

Patients with gastric epithelial neoplasia were consecutively enrolled between May 2010 and April 2012. The study was a prospective, randomized, multi-center, comparative trial. It was performed by 4 endoscopists in 4 institutions affiliated with The Catholic University of Korea (Incheon St. Mary's Hospital, St. Vincent's Hospital, Seoul St. Mary's Hospital, and Bucheon St. Mary's Hospital). All of the endoscopists were experienced with ESD and had performed ESD in over 200 cases. Before this study, two endoscopists (Kim BW, Lim CH) had used hook knife as the main device and the other two endoscopists (Chung WC, Kim TH) had used flex knife as the main device.

Adults (> 18 years) with histopathologic diagnosis of gastric epithelial neoplasia and without evidence of lymph-node involvement documented by abdominal computed tomography (CT) and/or endoscopic ultrasound (EUS) were included in this study. The lesions met the expanded criteria for local resection proposed by Gotoda<sup>[5,13,14]</sup>. Differentiated mucosal cancers of any size without ulceration or scarring, differentiated mucosal cancers < 30 mm in diameter with ulceration or scarring, or differentiated cancers with minimal submucosal invasion (< 500  $\mu$ m deep in the submucosa starting from the muscularis mucosae) were enrolled. The diameter of the lesions without ulcers was limited to a maximum of 60 mm. Patients with conditions that might have substantial effects on our study results (*e.g.*, serum creatinine > 2.5 mg/dL, total bilirubin > 3.0 mg/dL, platelet < 100000/mm<sup>3</sup>), patients who were consuming anti-platelet agents, patients with a history of previous gastric surgery and patients who did not consent to the study were excluded.

### Sample size

An estimated sample size of 37 subjects per group would give an 80% power to detect a difference in resection rate of the GTS compared to the HKC (assumed to have a complete resection rate of 90%), with a two-sided  $\alpha = 0.05$ . With a 10% drop out rate, 40 patients had to be recruited for each group.

### Randomization

All patients were randomly assigned to receive one of the knives-hook knife (Olympus; Tokyo, Japan) plus coagrasper (Olympus; Tokyo, Japan) or grasper type scissors (Alton Medical Instruments; Shanghai, China) (Figure 1) after evaluation with abdominal CT and/or EUS. Randomization codes (A-1 to A-10, B-1 to B-10) were packed into sealed opaque envelopes by an individual, who was not involved in screening and enrolment of the subjects to ensure concealment of allocation. In each of the study institute, twenty patients were enrolled.



Figure 1 Distal tip of the grasper type scissors. GTS has teeth inside the device and the outer side is not insulated. GTS: Grasper type scissors.

### Grasper type scissors

The diameter of the scissors is 2.4 mm and the serrated cutting edges are 4-mm-long. The outer side of the forceps except the tip is insulated so that electrosurgical current energy is concentrated at the blade to avoid burning the surrounding tissue. Unlike GSF, the tip of this knife is not insulated and has a thin cutting blade, so that it can facilitate the dissection of submucosal layer such as a flex knife. Furthermore, the forceps can be rotated to the desired location.

### Endoscopic submucosal dissection

A conventional gastroscope (GIF-Q240J or GIF-H260Z; Olympus, Tokyo, Japan) fitted with a transparent distal attachment (D-201-11304, Olympus) was used for the ESD procedures regardless of the ESD devices. Patients were sedated with intravenous midazolam (0.1 mg/kg) while in the endoscopic suite, and conscious sedation was maintained with additional injections during the procedure. After spraying indigo carmine dye, circumferential markings using argon plasma coagulation were made at 5 mm distances around the outside margin of the lesion, with 2 mm intervals between each marking dot. Hypertonic saline/epinephrine solution (1:10000) and indigo carmine mixture was injected into the submucosal layer until the mucosa was raised and additional injections were repeated as necessary during the procedure. After the lesion was lifted, mucosal incision was performed by using each type of knife and an electrosurgical generator (Erbe; Tübingen, Germany). Electrical current was set as endocut-I for hook knife and as endocut-Q for GTS. After incision around the lesion, dissection was conducted with either the hook knife or GTS (Figure 2).

Electrical current was set as forced coagulation for hook knife and as swift coagulation for GTS. When bleeding occurred during dissection, saline irrigation was performed. If the endoscopist performed the procedure with a hook knife, coagrasper was used for hemostasis according to the endoscopists' instructions, with an electrical current of 80 W for soft coagulation. With GTS, hemostasis was performed with an electrical current of

80 W for soft coagulation mode.

### Measurements

We compared the endoscopic appearance of tumors, location of tumors, *en bloc* resection rate, complete resection rate, size of resected specimens, histopathologic findings, operation time, and complication rates between the two groups. Complete resection was defined as lateral and vertical margins of the specimen being free from tumor involvement. Marking of the first dot and withdrawal of the endoscope were measured as the procedure time.

Bleeding was identified when melena, hematochezia, or the presence of fresh bloody vomitus along with a decreased hemoglobin levels of more than 2 g/dL were present after the resection. Perforation was identified by endoscopy during or just after the procedure and/or by the presence of intraperitoneal free air on plain abdominal radiography after the procedure.

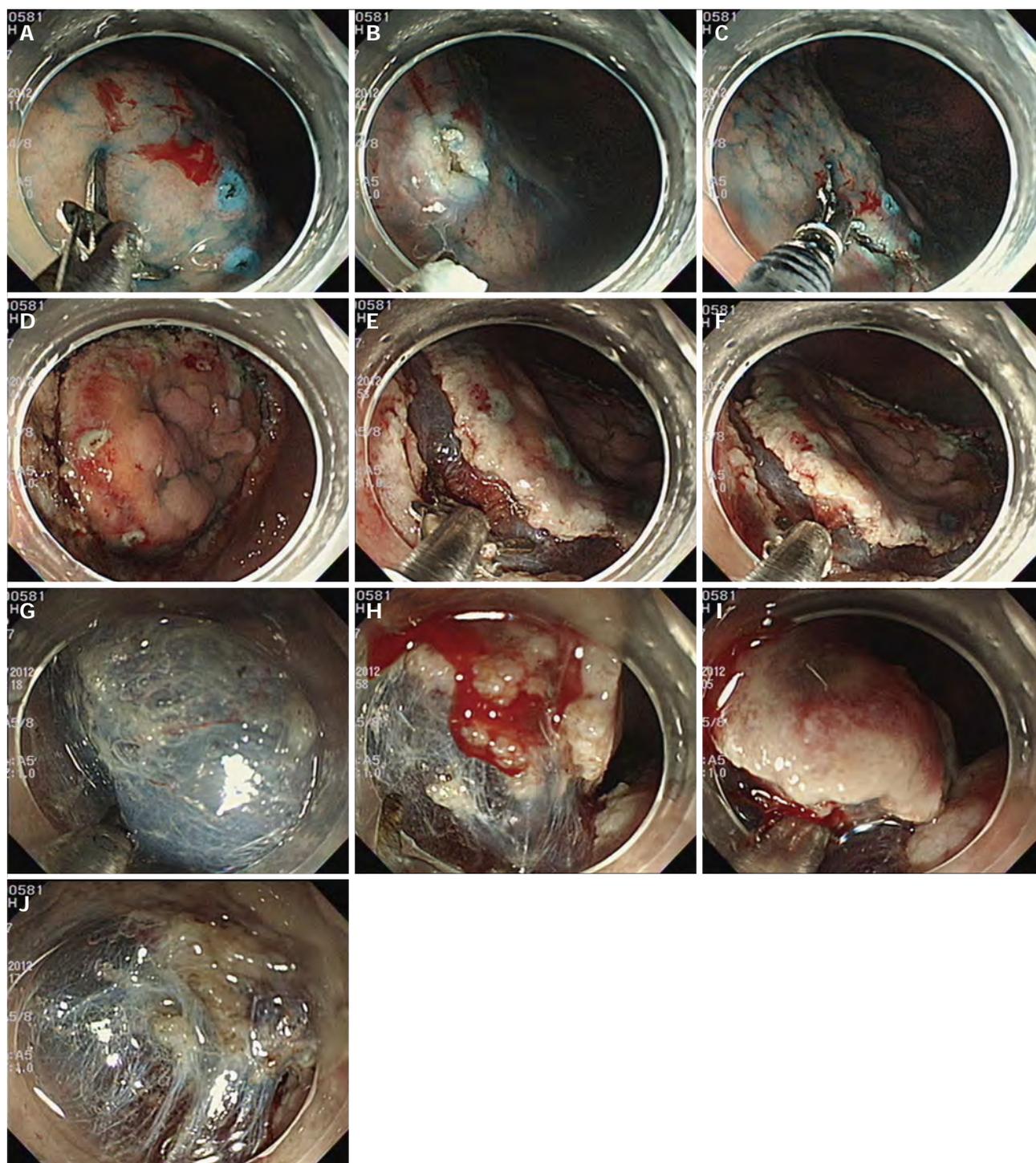
### Statistical analysis

All data were recorded on standard forms and computer analyzed. The Student *t* test was used to compare continuous variables between the groups. Differences between dichotomous variables were evaluated with the  $\chi^2$  test. The calculations were performed with the SPSS software (SPSS version 12.0, Chicago, IL, United States). Null hypotheses of no difference were rejected if *P* values were less than 0.05.

## RESULTS

A total of 78 patients were enrolled and 76 patients (37 patients of GTS knife group and 39 patients with HKC group) completed this study. Since over 37 patients in each group completed this protocol, additional enrollment was not conducted. One patient dropped out of the study because undifferentiated cancer was found in the resected specimen. Another patient with extensive submucosal infiltration ( $> 500 \mu\text{m}$  deep in the submucosa starting from the muscularis mucosae) was also dropped out of the study. Both patients underwent additional surgery after ESD.

The mean age of the GTS group was  $62.3 \pm 11.4$  years and mean age of the HKC group was  $65.6 \pm 10.1$  years (Table 1). There was no significant difference in age and sex ratio between the two groups. Differentiated adenocarcinoma was found in 32.4% in the GTS group and 33.3% in the HKC group. The pathologic distribution and mean size of the resected specimens were not different between the two groups. The area of the resected specimens was  $8.30 \pm 4.52 \text{ cm}^2$  in GTS group and  $8.59 \pm 4.43 \text{ cm}^2$  in HKC group. The depth of tumor distribution was not different between the two groups (94.6% of mucosal layer in the GTS group, 92.3% in the HKC group). With regard to tumor location, 64.9% (24/37 cases) were located at the antrum in the GTS group and 71.8% (28/39 cases) in the HKC group. The locations were not significantly different between the two groups. The total time



**Figure 2** Procedure of endoscopic submucosal dissection with distal tip of the grasper type scissors. A: Grasper type scissors (GTS) is ready for puncture; B: Puncture was completed with GTS; C: Incision is conducted with GTS; D: Complete incision was performed with GTS; E: Submucosal dissection is ready with GTS; F: Submucosal dissection is conducted by grasping the submucosal layer; G: Submucosal dissection is conducted with blade of the GTS just like a flex knife; H: Hemorrhage from the submucosal layer is noted; I: Bleeding focus was grasped with GTS; J: Bleeding was controlled after coagulation with GTS.

elapsed during the procedure was  $44.54 \pm 21.72$  min in the GTS group and  $43.77 \pm 21.84$  min in the HKC group ( $P = 0.88$ ). The time elapsed per square centimeter of the resected lesion was  $7.53 \pm 6.35$  min/cm<sup>2</sup> in the GTS group and  $6.92 \pm 5.93$  min/cm<sup>2</sup> in the HKC group ( $P = 0.66$ ). The *en bloc* resection rate was 100% in both groups. The overall complication rate was 5.41% (2/37 cases)

in the GTS group and 7.69% (3/39 cases) in the HKC group ( $P = 0.68$ ).

In Korea, the prices of the knives used for ESD are the same. GTS and hook knife cost about 199240 Won (about 185 dollars) and coagrasper cost about 210000 Won (about 191 dollars). HKC are about double in cost compared to GTS alone.

**Table 1** Characteristics of the enrolled patients *n* (%)

Characteristics	GTS	HKC	<i>P</i> value
Number of patients (total)	37	39	0.30
Operator 1 (Kim BW)	10	10	
Operator 2 (Chung WC)	9	10	
Operator 3 (Lim CH)	8	9	
Operator 4 (Kim TH)	10	10	
Male:female	26:11	23:16	0.19
Age (mean ± SD, yr)	62.3 ± 11.4	65.6 ± 10.1	0.58
Final pathologic diagnosis			0.68
Tubular adenoma, low grade dysplasia	22	20	
Tubular adenoma, high grade dysplasia	3	6	
Adenocarcinoma, well differentiated	6	8	
Adenocarcinoma, moderately differentiated	6	5	
Location of the lesion			
Antrum	24	28	
Angle	5	3	
Body	8	8	
Long axis (cm)	3.59 ± 1.10	3.68 ± 0.98	0.70
Short axis (cm)	2.75 ± 0.83	2.79 ± 0.84	0.83
Area (cm <sup>2</sup> )	8.30 ± 4.52	8.59 ± 4.43	0.78
Total elapsed time (mean ± SD, min)	44.54 ± 21.72	43.77 ± 21.84	0.88
Elapsed time/cm <sup>2</sup> (mean ± SD, min/cm <sup>2</sup> )	7.53 ± 6.35	6.92 ± 5.93	0.66
<i>En bloc</i> resection	37/37 (100)	39/39 (100)	
Incomplete resection	0/37 (0)	0/39 (0)	
Complications	1/37 (2.7)	2/39 (5.1)	
Perforation	1	1	
> 2.0 g/dL Hb decrease 1 d after procedure	0	1	

GTS: Grasper type scissors; HKC: Hook knife plus coagrasper.

## DISCUSSION

Conventional EMR was previously recommended as a curative local treatment for early gastric cancer<sup>[15,16]</sup>. To achieve curative resection with adequate margins, the excisions need to be large enough. When piecemeal resection occurs, electro-cautery across the specimens could affect the accuracy of assessment of the lateral margins and this could result in a higher risk of local recurrence. ESD is intended to perform large mucosal resections and improves the rate of successful *en bloc* resection of an early stage gastrointestinal neoplasia<sup>[17,18]</sup>. To date, novel devices have been developed for the completion of *en bloc* resection with adequate margins. However, many endoscopists are eager for the development of devices that allow more effective and faster procedures. In this study, we aimed to introduce and to evaluate the efficacy and technical aspects of the GTS.

Since GSF is ideally designed for both incising the targeted tissue and hemostasis, we tried to improve the device by improving its effect during dissection. Theoretically, the advantage of GSF for ESD is that the device can prevent unexpected incisions. By elevating the lesion during dissection, GSF provides good visualization of

the submucosal layer. However, GSF has the disadvantage that it cannot be opened when using a conventional cap because of the small cap diameter. Therefore, a special hood is required when using the GSF. We modified the GSF and developed the GTS, which can accurately grasp and incise the targeted tissue using electrosurgical current. It is smaller than GFS so that it can be used with the conventional transparent cap. The most important difference between GTS and GSF is that the tip of GTS is not insulated and has a thin cutting blade, so that it can facilitate the dissection of the submucosal layer.

Most of the knives are designed for cutting or dissection, but they are not adequate for hemostasis during the ESD procedure. The use of additional instruments increases the cost and requires more procedure time because it takes time to change the instruments. The scissor-type device makes it possible to perform dissection and hemostatic procedures without changing the devices. In this study, we compared the novel GTS, which can be used for both dissection and hemostasis, with HKC for ESD of gastric epithelial neoplasia. We compared these knives because HKC is one of the most commonly used devices for ESD in Korea. In our results, the elapsed time did not differ significantly between GTS and HKC and the procedure time was not saved by using the GTS. This result may have been caused by various factors such as the fact that the endoscopists of this study were not experienced with the new instrument. Furthermore, 2 of the endoscopists had been using the HKC and were familiar with this device which may have affected the procedure time. It is known that there is a learning curve for ESD and that experience of the procedure shortens the procedure time<sup>[19,20]</sup>. We believe that increased experience with the GTS will reduce the procedure time. Nonetheless all the lesions were removed successfully with the GTS by the 4 endoscopists, which suggest that any experienced endoscopists can complete the whole ESD procedure with the GTS. Feasibility of ESD for gastric epithelial neoplasia with the GTS in beginners should be elucidated in the future.

One case of perforation occurred in the GTS group which might have been prevented if a knife with an insulated body such as GSF was used. The perforation rates of ESD while using different types of knives were reported to be 4%-10%<sup>[21-24]</sup>, which is similar to the perforation rates of our result. A decrease in hemoglobin level over 2 g/dL per day after the procedure was the same in both groups. Thermal and mechanical tissue damage at the GTS-tissue interface was expected because GTS is larger compared to the hook knife. However, contrary to our expectations, GTS did not interfere with the pathologists' interpretation of the specimens and complete resections with adequate margins were obtained pathologically with GTS.

There are some limitations in this study. We designed this device to reduce the procedure time, but the sample size was limited and we could not figure it out. Four experienced endoscopists participated in this study and the

procedure times by beginners also should be examined. Further studies with larger sample sizes are anticipated to show other benefits of this device.

In conclusion, ESD for gastric epithelial neoplasia can be performed with GTS alone regardless of size and location of the lesion when the endoscopists are experienced. GTS is a safe and effective device for ESD of gastric epithelial neoplasia when compared to HKC. ESD can be performed with GTS alone, which can reduce the costs. Further experiences and large scaled studies would be anticipated to compare the various devices.

## COMMENTS

### Background

Various cutting devices have been developed for endoscopic submucosal dissection (ESD). Most of these knives are designed for dissection of the submucosa and sometimes are not adequate for control hemorrhages developed during the procedure. The authors recently designed a new device, grasper type scissors (GTS) both for dissection and control hemorrhages.

### Research frontiers

Grasping-type scissors forceps (GSF) was developed by Akahoshi *et al* for dissection and control hemorrhages. However, it requires a special hood and rotating the device is frequently difficult because of the size. The safety and effectiveness of GSF was performed in a single center.

### Innovations and breakthroughs

The most important difference between GTS and GSF is that the tip of GTS is not insulated and has a thin cutting blade, so that it can facilitate the dissection of the submucosal layer. The safety and effectiveness of GTS was evaluated in multi-centers in this study.

### Applications

This study suggests that ESD for gastric epithelial neoplasia can be performed with GTS alone regardless of size and location of the lesion when the endoscopists are experienced.

### Peer review

The authors developed a novel device which was modified from GSF, GTS, for ESD of gastric neoplasia. In this study, GTS resolved several disadvantage of GSF and clearly succeeded to reduce the cost of ESD. This study is very interesting, exciting and useful for all endoscopists.

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## Isolation and biochemical analysis of vesicles from taurohyodeoxycholic acid-infused isolated perfused rat livers

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### Abstract

**AIM:** To isolate biliary lipid-carrying vesicles from isolated perfused rat livers after taurohyodeoxycholic acid (THDC) infusion. Biliary lipid vesicles have been implicated in hepatic disease and THDC was used since it increases biliary phospholipid secretion.

**METHODS:** Rat livers were isolated and perfused *via* the hepatic portal vein with THDC dissolved in Krebs Ringer Bicarbonate solution, pH 7.4, containing 1 mmol/L CaCl<sub>2</sub>, 5 mmol/L glucose, a physiological amino acid mixture, 1% bovine serum albumin and 20% (v/v) washed human erythrocytes at a rate of 2000 nmol/min for 2 h. The livers were then removed, homogenized and subjected to centrifugation, and the microsomal fraction was obtained and further centrifuged at 350000 *g* for 90 min to obtain subcellular fractions. These were analyzed for total phospholipid, cholesterol, protein and alkaline phosphodiesterase I (PDE).

**RESULTS:** No significant changes were observed in the total phospholipid, cholesterol and protein contents of the gradient fractions obtained from the microsomal preparation. However, the majority of the gradient fractions ( $\rho = 1.05-1.07$  g/mL and  $\rho = 1.95-1.23$  g/mL) obtained from THDC-infused livers had significantly higher PDE activity compared to the control livers. The low density gradient fraction ( $\rho = 1.05-1.07$  g/mL) which was envisaged to contain the putative vesicle population isolated from THDC-perfused livers had relatively small amounts of phospholipids and protein when compared to the relevant control fractions; however, they displayed an increase in cholesterol and PDE activity. The phospholipids were also isolated by thin layer chromatography and subjected to fractionation by high performance liquid chromatography; however, no differences were observed in the pattern of the fatty acid composition of the phospholipids isolated from THDC and control perfused livers. The density gradient fractions ( $\rho = 1.10-1.23$  g/mL) displayed an increase in all the parameters measured from both control and THDC-infused livers.

**CONCLUSION:** No significant changes in biliary lipids were observed in the fractions from THDC-infused livers; however, PDE activity was significantly increased compared to the control livers.

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**Key words:** Taurohyodeoxycholic acid; Phospholipids; Biliary cholesterol; Bile; Vesicles

**Core tip:** Bile contains various constituents including cholesterol and phospholipid, mainly phosphatidylcholine with a unique fatty acid composition of 1-palmitoyl 2-linoleyl (16:0-18:2) phosphatidylcholine and 1-palmitoyl 2-oleoyl (16:0-18:1). These biliary lipids are transported in vesicles from a specific intra-hepatic pool and an increase in biliary lipid-carrying vesicles may have

implications for hepatic diseases such as gallstone formation. Taurohyodeoxycholic acid (THDC) stimulates the secretion of biliary phospholipids; hence THDC-infused rat livers were subjected to ultracentrifugation in order to isolate these phospholipid-carrying vesicles. The isolation of these biliary lipid-carrying vesicles was not successful; however, vesicles enriched in PDE activity were obtained.

Hismiogullari AA, Hismiogullari SE, Rahman K. Isolation and biochemical analysis of vesicles from taurohyodeoxycholic acid-infused isolated perfused rat livers. *World J Gastroenterol* 2013; 19(37): 6228-6236 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6228.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6228>

## INTRODUCTION

Phospholipids and cholesterol are synthesized in the hepatocytes and are thought to be transferred into bile by vesicular and non-vesicular mechanisms. Biliary lipids mainly consist of cholesterol and phospholipids and their secretion into bile is effected by secretion of bile salts<sup>[1]</sup>. Hepatocytes acquire biliary lipid by three pathways namely biosynthesis, lipoproteins and existing lipid molecules drawn from intracellular membranes and newly synthesized biliary lipids; these account for less than 20% of the total lipids<sup>[2]</sup>.

The majority of biliary phospholipid is phosphatidylcholine (PC) with distinct fatty acid composition, namely 1-palmitoyl 2-linoleyl (16:0-18:2) PC and 1-palmitoyl 2-oleoyl (16:0-18:1), whereas hepatocyte PC contains significant amounts of different phospholipid classes<sup>[3,4]</sup>. Very few studies have been performed on biliary lipid transport in hepatocytes<sup>[5,6]</sup>, whereas numerous studies have been performed on bile, its formation and composition especially related to lipids, and these studies have identified several physical forms of lipid carriers, including biliary vesicles<sup>[7,8]</sup>.

The main source of biliary lipid, before its appearance in bile, has been suggested to be the bile canalicular membrane where it is removed by the detergent action of bile salts. These lipids are then thought to be continuously replaced from within the cell, probably *via* vesicular transport, for biliary lipid secretion to continue without damage to the liver and canalicular membrane<sup>[9]</sup>. In support of this, inhibitors of microtubular function such as colchicine and vinblastine have been shown to reduce biliary lipid secretion<sup>[10-12]</sup>. Such vesicles supplying lipids to the plasma membrane have also been shown and isolated in other cell types, thus, it can be postulated that biliary lipid is probably supplied to the canalicular membrane *via* such vesicles. This is supported by the fact that increased numbers of vesicles have been observed accumulating near the bile canaliculus during extensive bile acid secretion<sup>[2,9,13-15]</sup>. The isolation of putative biliary lipid-carrying vesicles, however, is difficult due to the wide range of

vesicle types in hepatocytes, and the difficulty of identifying them because of inadequate criteria.

Hepatic ATP-binding cassette half-transporter genes 5/8 (*ABCG5* and *ABCG8*) are expressed in the canalicular membrane of hepatocytes and have an essential role in biliary cholesterol secretion<sup>[16-19]</sup>. However, the pathways involved in trans-hepatic cholesterol trafficking into bile are still not clear and a specific cholesterol transport protein has not been confirmed in hepatocytes. Biliary cholesterol secretion is important for the two important disease complexes of atherosclerotic cardiovascular disease (CVD) and gallstone disease<sup>[1]</sup>. In atherosclerotic CVD, biliary cholesterol secretion is thought to be the final step in the completion of the reverse cholesterol transport pathway which includes the transport of peripheral cholesterol back to the liver for excretion into bile. Increase in biliary cholesterol secretion can lead to the supersaturation of bile and under the right conditions this may lead to the formation of cholesterol gallstones<sup>[1]</sup>. Taurohyodeoxycholic acid (THDC) is a natural 6 $\alpha$ -hydroxylated bile acid with hydrophilic properties, causing more secretion of PC into bile compared to taurosoxycholic acid and taurocholic acid, whereas no significant differences were found in the biliary secretion of cholesterol<sup>[20]</sup>. Due to its relatively high hydrophilicity, THDC has been proposed for use instead of other bile acids for the treatment of cholesterol gallstone dissolution<sup>[21]</sup>. Angelico *et al.*<sup>[20]</sup> showed by the use of electron microscopy that increased recruitment of vesicles and lamellar bodies around and within bile canaliculi in the liver occurred with THDC infusion. The identification of biliary lipid-carrying vesicles may have implications for the treatment of hepatic disorders such as cholesterol gallstone formation.

The aim of this study was to isolate these biliary lipid-carrying vesicles in hepatocytes by using a novel gradient centrifugation technique and to verify their origin by separating PC by thin layer chromatography (TLC) and measuring its unique fatty acid pattern by high-performance liquid chromatography (HPLC). Cholesterol was measured by gas liquid chromatography (GLC).

## MATERIALS AND METHODS

### Chemicals

All chemicals were purchased from Sigma Chemical Co., Poole, Dorset, United Kingdom, except for cannulation tubing PP10 (internal diameter 0.28 mmol/L) which was obtained from Portex Ltd., Hythe, United Kingdom.

### Animals and treatments

Animals used throughout this study were male Wistar rats (250-300 g), bred within Liverpool John Moores University, Life Services Support Unit, and they were allowed free access to standard laboratory diet in powdered form.

### Perfusion of isolated liver

Rats were anaesthetized with sodium pentobarbitone (6 mg/100 g body weight, intraperitoneally) before starting

the experiment. Once isolated, livers were perfused in the absence and presence of THDC infusion *in situ* using the method of Rahman and Coleman<sup>[22]</sup>. Heparin (2500 units/0.5 mL) was injected into the vena cava and after 2 min, the hepatic portal vein was cannulated with a Wallace 17.5 G cannula and the perfusion was commenced immediately with 150 mL of Krebs ringer bicarbonate buffer, pH 7.4, containing 1 mmol/L CaCl<sub>2</sub>, 5 mmol/L glucose, a physiological amino acid mixture, 1% bovine serum albumin and 20% (v/v) washed human erythrocytes, and the abdominal aorta was severed. The inferior vena cava was then cannulated with a Wallace 16 G cannula and a recycling perfusion commenced by returning the efferent perfusate to the original perfusate pool which was gassed continuously with O<sub>2</sub>/CO<sub>2</sub> (19:1, v/v). The livers were maintained in a thermostatically controlled cabinet at 37 °C throughout the experiment. As soon as the liver perfusion was established, THDC infusion was commenced into the hepatic portal cannula at a rate of 2000 nmol/min for 2 h to stimulate delivery of lipid-carrying vesicles to the canalicular membrane.

### Liver homogenization

At the end of perfusion livers were removed, weighed and transferred to 3 vol. (w/v) of ice-cold buffered sucrose (0.25 mol/L containing 1 mmol/L HEPES pH 7.4). They were then cut into several large pieces and swirled around in the buffer to remove as much blood as possible. The livers were then minced finely with sharp scissors, transferred to an ice-cold homogenizing vessel and were finally homogenized with about six strokes of the pestle at full speed. Finally, the homogenate was made up to 4 vol. (w/v) with sucrose buffer solution.

### Fractionation of liver homogenate

The homogenate from the liver was used to produce subcellular fractions based on the method of Ford and Graham<sup>[23]</sup>. A sample of homogenate (3-4 mL) was removed for analysis and the remainder was centrifuged in a fixed angle rotor at 4 °C for 10 min at 1000 *g* to pellet the nuclei and heavy mitochondria. The pellet was then suspended in sucrose buffer and stored frozen at -20 °C until analysis.

Further centrifugation was performed at 4000 *g* for 10 min to produce the mitochondrial fraction, followed by 15000 *g* for 20 min to produce the light mitochondrial and lysosome fraction. A final centrifugation step at 100000 *g* for 45 min was then performed and the microsomal fraction was obtained. All fractions were assayed for cholesterol, phospholipids, protein and PDE activity

### Purification of vesicles from microsomal fraction

The microsomal pellet was then dissolved in sucrose buffer solution up to 8 mL and then loaded onto 2 mL of OptiPrep<sup>TM</sup> (1.32 g/mL) in a Beckman Vti65 vertical tube rotor and centrifuged at 350000 *g* for 90 min at 4 °C. At the end of the centrifugation, the gradient was fractionated by upward displacement into 10 × 1 mL samples and these fractions were analyzed for cholesterol, phos-

pholipids, protein and PDE activity.

### Lipid analysis

Cholesterol was analyzed by GLC as trimethylsilyl ether derivatives as described by Zak *et al.*<sup>[24]</sup>. Phospholipid was extracted from the liver fractions as described by Bligh and Dyer<sup>[25]</sup> in a method by which lipid is extracted into a chloroform-methanol-water mixture. Addition of further chloroform and water forms a biphasic system with non-lipids passing into the methanol-water phase. The phospholipid in the chloroform phase was then assayed by the method of Bartlett<sup>[26]</sup> in which organic phosphate is digested and the resulting orthophosphate is determined by converting it to phosphomolybdic acid, which is reduced to a blue complex allowing spectrophotometric measurement at 830 nm.

### Alkaline phosphodiesterase I analysis

PDE (EC 3.1.4.1) was measured at 37 °C, essentially as described by Trams and Lauter<sup>[27]</sup>.

### Thin layer chromatography of phospholipids

**Sample extraction:** 200 µL of sample was added to 200 µL of distilled water in a 2 mL (microcentrifuge) tube followed by the addition of 750 µL of chloroform then methanol (1:2 v/v) to each tube, vortex mixed and left to stand for 20 min. After this time, 250 µL of chloroform and 250 µL of distilled water were added and the tubes were vortex mixed and then centrifuged for 1 min. The lower organic phase was then transferred to a clean tube and placed in a water bath at 37 °C to evaporate the chloroform<sup>[25]</sup>. Samples were finally redissolved in 30 mL of chloroform, vortex mixed and loaded on to the TLC plates.

**Solvent for running phospholipid plates:** The plates were developed in a solvent mixture containing chloroform/methanol/glacial acetic acid/water (75:45:12:1.5 by volume). These solvent ratios were poured into a tank containing a filter paper layered against the wall of the chamber and the lid was replaced; the tank was then left to saturate for at least 30 min prior to running the plates. The plates were left to run until solvent reached the scored solvent front line (approximately 75 min) and were then removed and air dried in a fume cupboard.

**Developing the TLC plates:** The dried plates were developed in an iodine tank and the position of the phospholipid was marked with a needle. The PC bands were scraped and the silica transferred to extraction tubes; at the same time silica was scraped from a similar area without any phospholipids to act as a control. Phospholipids were extracted with 2 × 1 mL methanol (HPLC grade) and vortex mixed for 5 min, vortexed again, centrifuged and the methanol extract was then transferred to clean glass tubes and dried at 37 °C under nitrogen. The dried lipids were redissolved in 1 mL of methanol (HPLC grade) and 2 × 10 µL aliquots were removed for phospholipids assay. The remainder was filtered, dried at 37 °C

under nitrogen and stored cool and in the dark until required for HPLC analysis. At least 20 nmol of PC was injected onto the HPLC column.

### HPLC

HPLC analysis was performed using a Bio-Rad HPLC 2700, Series 8000 Gradient System V 2.30.1a liquid chromatograph equipped with an oven column module, and a spectrophotometric detector, Bio-Rad Model 1801 UV monitor. The sample was injected onto the column by a Rheodyne injector equipped with a 20  $\mu$ L sample loop. An HPLC column of 100 mmol/L  $\times$  4.6 mmol/L *id*, packed with a Spherisorb ODS2 bonded phase, and with a 3  $\mu$ L particle size was used and the mobile phase consisted of 20 mmol/L choline chloride in methanol/water/acetonitrile (90:8:3, by vol.). The operating conditions were: column temperature, 60  $^{\circ}$ C; chromatographic profile: initial flow, 1 mL/min, held for min and then a linear increase to 2 mL/min over 20 min; the final flow of 2 mL/min being held for 10 min.

### Protein estimation

Protein estimation was determined by the method of Winterbourne and all samples were assayed in duplicate<sup>[28]</sup>. A sheet of 3 mmol/L Whatman chromatography paper was divided into 1 cm  $\times$  1 cm squares. Standard protein concentration BSA ranged from 0.5-8 mg/mL and the standards were prepared by spotting corresponding amounts onto the center of the squares on the sheet. 3  $\mu$ L of sample was carefully spotted onto the center of individual squares and blank squares were left for the determination of background staining. The standards and samples were then left to dry and were later fixed by immol/Lersing into 10% TCA solution for 15 min and the sheet was then transferred to a working dye solution (0.04% w/v Coomassie Blue, 25% v/v ethanol and 12% v/v acetic acid) and left to stain for 1 h. The sheet was then destained by immersing in three changes of destaining solution 10% (v/v) methanol and 5% (v/v) glacial acetic acid for 10 min, and it was then left to dry in the oven at 80  $^{\circ}$ C. The grid was then cut into its individual component squares and was placed into small plastic vials containing 1 mL eluent, 1 mol/L potassium acetate in 70% (v/v) ethanol for 1 h and finally the absorbance of the eluted dye solution was read at 590 nm against the background dye measurements.

### Statistical analysis

Data were subjected to 2-tailed paired *t* test and *P* values  $\leq$  0.05 were considered as statistically significant.

## RESULTS

### Total phospholipids and total cholesterol in the subcellular fraction of isolated perfused rat livers in the absence and presence of THDC infusion

Total phospholipids and cholesterol in the subcellular fractions from isolated perfused rat livers in the absence

and presence of THDC infusion are shown in Figure 1A and B. The total lipids have been expressed as  $\mu$ mol/g of liver due to the differences in liver weight of the animals. No significant differences were found in total phospholipids and cholesterol content of subcellular fractions from control and THDC-infused rat livers (Figure 1A and B).

### Total protein and PDE activity in subcellular fractions of liver homogenate

Total protein and PDE activity in subcellular fractions of isolated perfused rat livers in the absence or presence of THDC infusion are depicted in Figure 1C and D. No significant differences were found in total proteins in the subcellular fractions of isolated perfused rat livers in the absence or presence of THDC infusion (Figure 1C). However, PDE activity was significantly higher in fraction P3 II from THDC-infused livers (Figure 1D).

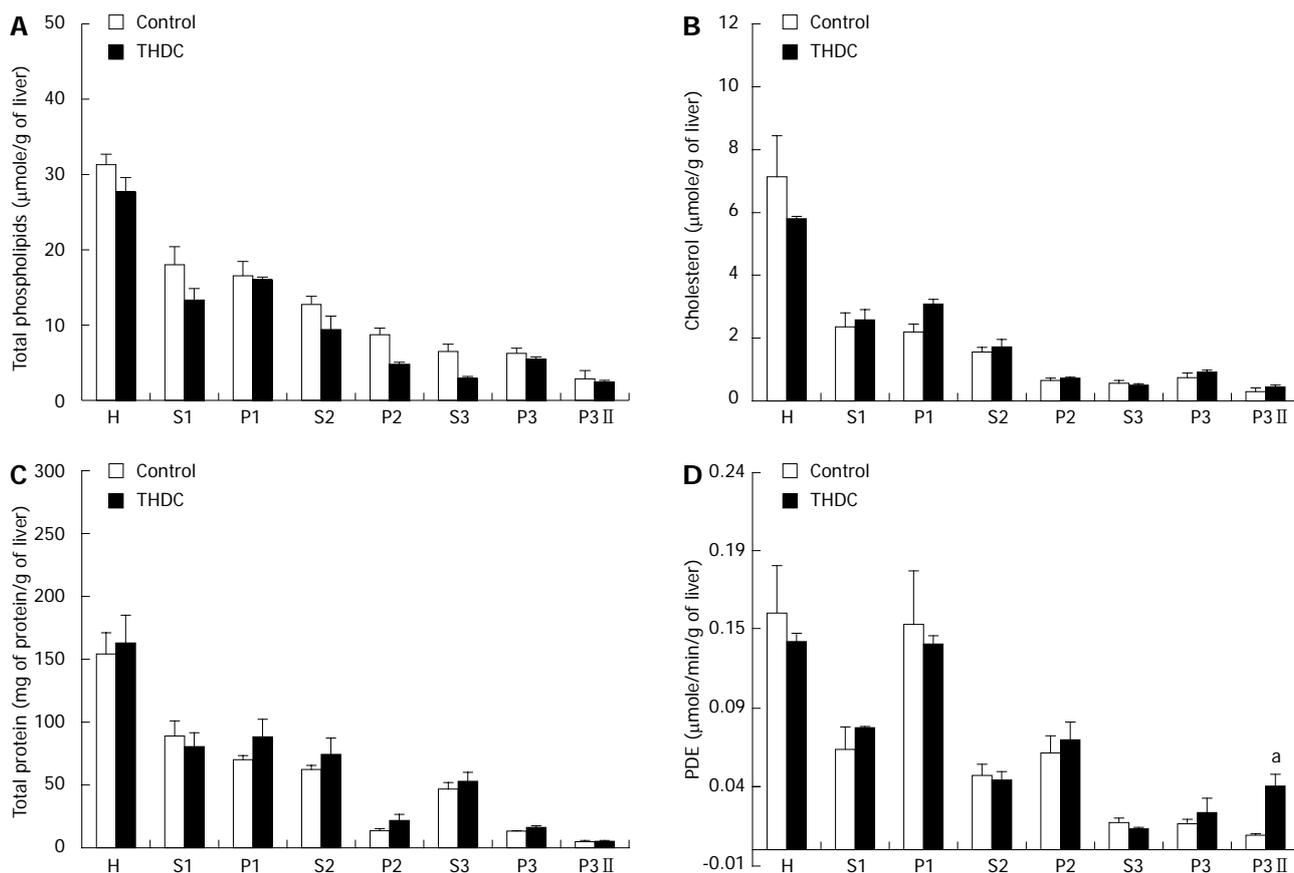
### Analysis of the subfractions of the microsomal fraction

The microsomal fractions were subjected to self-generating density gradient centrifugation and the fractions were analyzed for total phospholipids, cholesterol, protein and PDE activity. The results are presented in Figure 2. Enzyme activity in THDC-infused liver fractions 1, 2, 8, 9 and 10 was significantly higher when compared to the control values. However, no significant differences were observed in total phospholipids, cholesterol and protein in the subcellular fractions of isolated perfused rat livers in the absence or presence of taurohyodeoxycholic acid infusion.

Biliary PC has a unique fatty acid pattern, 1-palmitoyl 2-linoleyl (16:0-18:2) PC, 1-palmitoyl 2-oleoyl (16:0-18:1) PC, which is distinct from that of membrane PC fatty acid pattern 1-stearoyl 2-arachidonyl (16:0-20:4). No differences in the level of subfractions of PC were found (Table 1). It was not possible to identify peak 1 due to lack of relevant standards (Figure 3).

## DISCUSSION

The liver is the site of many important biochemical functions including formation of bile which contains many solutes including phospholipids and cholesterol, both of which are synthesized in the liver and have been implicated in liver and cholestatic disease<sup>[29]</sup>. Many studies have been performed in which the physical forms of lipids have been isolated in bile; however, very few studies have addressed this problem in the liver. Attempts to isolate vesicles containing biliary type PC and cholesterol have largely been unsuccessful in hepatocytes<sup>[5]</sup>. However, Gilat and Sömjen<sup>[7]</sup> and Sömjen *et al*<sup>[8]</sup> have identified three forms of biliary lipid carriers in bile, namely unilamellar vesicles, stacked lamellae and micelles. The sources of biliary phospholipids may be numerous: *de novo* synthesis, microsomes, Golgi, bile canalicular membrane and preformed hepatic and extrahepatic pool<sup>[30]</sup>. The extrahepatic pool may contribute about 40% of the biliary phospho-



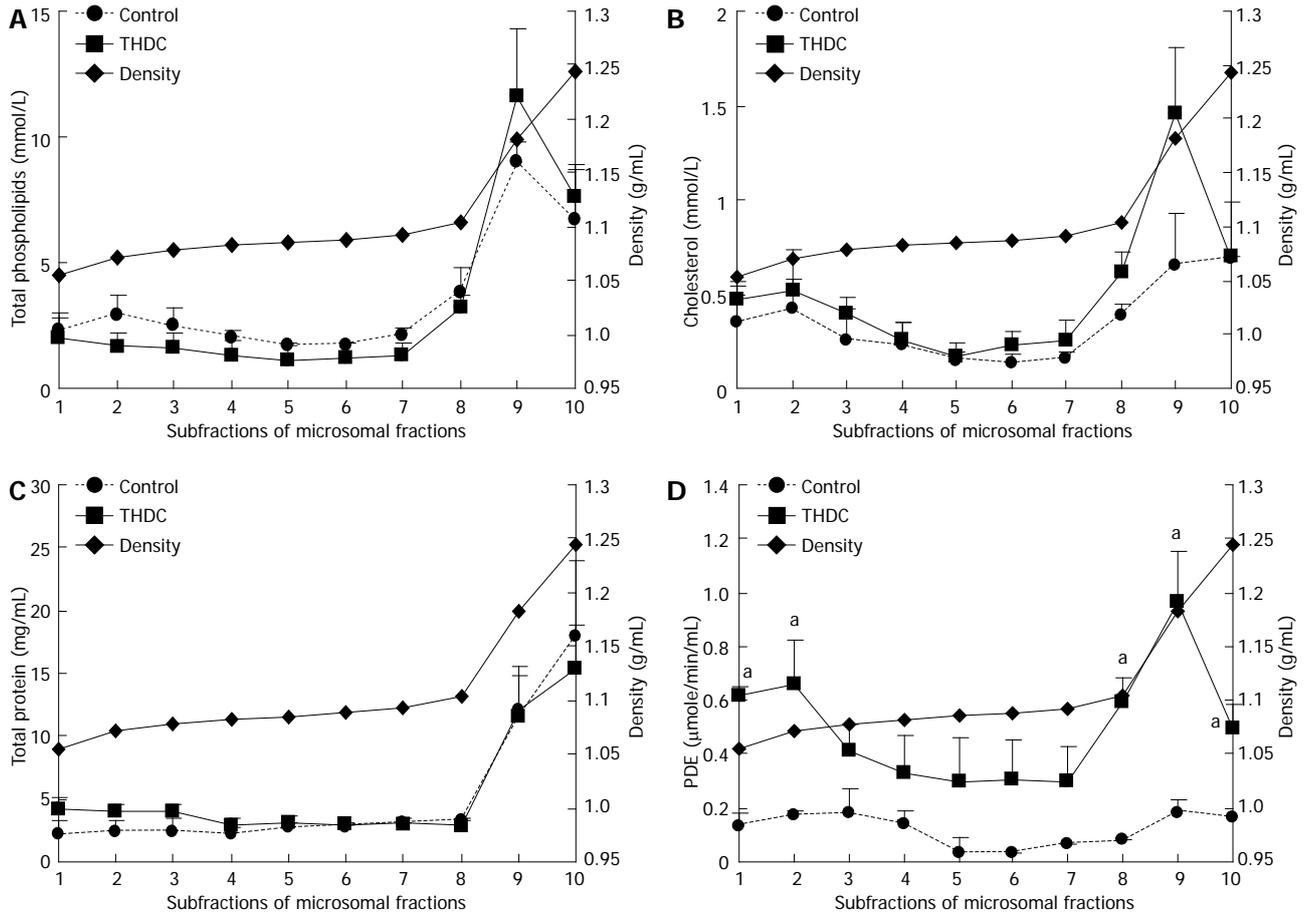
**Figure 1** Total phospholipids, cholesterol, protein and phosphodiesterase I activity in subcellular fractions of liver homogenate. A: Phospholipids; B: Cholesterol; C: Protein; D: Phosphodiesterase I activity (PDE). THDC: Taurohyodeoxycholic acid. Symbols are homogenate (H), supernatant 1 (S1), pellet 1 (P1), supernatant 2 (S2), pellet 2 (P2), supernatant 3 (S3), pellet 3 (P3) and pellet 3 (P3 II) diluted with sucrose and KCl. Results are presented as mean  $\pm$  SE ( $n = 6$ ). Significant differences from controls were assessed by student's *t*-test and are indicated by (<sup>a</sup> $P < 0.05$  vs control group).

lipids secreted in the basal state in rats and is associated with high density lipoprotein<sup>[31]</sup>, also bile acid activates a specific cytosolic PC transfer protein in the hepatocytes which then transfers PC to the canalicular membrane. It has also been reported that a PC transmembrane translocator (*flippase*) exists in the canalicular membrane and may be involved in the membrane translocation of specific PC to the biliary side of the canalicular membrane<sup>[32]</sup>. Most of the cholesterol secreted in bile is derived from circulating plasma lipoprotein, mainly low-density lipoprotein, high-density lipoprotein and chylomicron remnants. Cholesterol is transported probably in vesicles and binds to protein such as sterol carrier protein 2 present in the hepatocytes and, under physiological conditions, biliary bile acid secretion is the driving force behind the secretion of phospholipid and cholesterol in bile<sup>[33,34]</sup>.

THDC is a hydrophilic bile acid, causing more secretion of biliary PC compared to tauroursodeoxycholic acid and taurocholic acid, whereas this bile acid does not significantly increase biliary secretion of cholesterol and protein when compared to the control<sup>[20]</sup>. It was thought that increased biliary PC carrier vesicles would be present in the hepatocytes in this experiment. This concept was initiated by the study of Angelico *et al*<sup>[20]</sup>, who observed by electron microscopy that increased recruitment

of vesicles and lamellar bodies around and within bile canaliculi in the liver occurred with THDC infusion. It is possible that mechanisms at a molecular level include stimulation by THDC of the PC transfer protein and/or of the phospholipid translocator involved in the transmembrane canalicular transport of phospholipids. It has also been reported from physical-chemical and imaging studies that bile salts stimulate the biliary secretion of unilamellar vesicles from the external hemileaflet of the canalicular membrane<sup>[31]</sup>.

However, no significant differences were observed between control and THDC-infused rat liver sub-fractions in total phospholipids and cholesterol (Figure 1A and B). There was no significant difference in total proteins in the subcellular fractions of isolated perfused rat livers in the absence of THDC infusion (Figure 1C). However, PDE activity in the subcellular fraction P3II was significantly higher than in the corresponding fraction from the control experiment. Within the liver, PDE is a membrane-bound enzyme and would be expected to be associated with vesicles, hence this enzyme was assayed and results may indicate that there is more membrane material in this fraction. This is in contrast to the results reported by Lanzarotto *et al*<sup>[35]</sup> who observed that chronic administration of THDC in humans with intact



**Figure 2** Analysis of the subfractions of the microsomal fraction. A: Phospholipids; B: Cholesterol; C: Protein; D: Phosphodiesterase I activity (PDE). Livers were removed and the microsomal fraction was prepared and subjected to centrifugation and sub-fractions obtained. The sub-fractions were analyzed for total phospholipids, total cholesterol, protein and PDE activity. Results are presented as mean  $\pm$  SE ( $n = 6$ ). THDC: Taurohydoxychoic acid. Significant differences from controls were assessed by student's *t* test and indicated by <sup>a</sup> $P < 0.05$  vs control group.

**Table 1** High-performance liquid chromatography analysis of the fatty acid composition of phosphatidylcholine

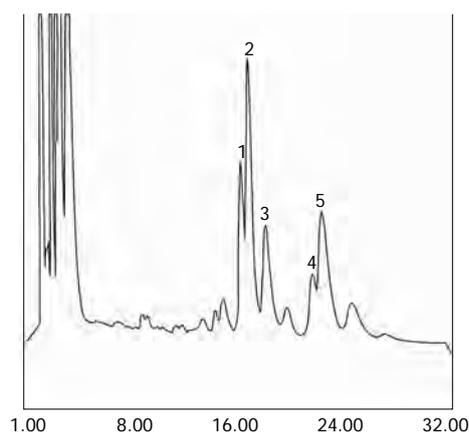
Fractions	PC (nmol)	C16:0 C20:4	C16:0 C18:2	C16:0 C18:1
Control perfused fed rat livers				
1	0.171 $\pm$ 0.001	41.147% $\pm$ 3.02%	11.048% $\pm$ 0.8%	8.327% $\pm$ 0.92%
2	0.223 $\pm$ 0.003	42.219% $\pm$ 2.8%	10.223% $\pm$ 0.7%	6.49% $\pm$ 0.5%
8	0.214 $\pm$ 0.007	41.545% $\pm$ 3.09%	11.281% $\pm$ 0.8%	6.436% $\pm$ 0.9%
9	1.02 $\pm$ 0.05	43.59% $\pm$ 1.5%	8.68% $\pm$ 1%	6.61% $\pm$ 0.73%
10	1.34 $\pm$ 1.1	44.64% $\pm$ 0.27%	8.19% $\pm$ 0.64%	6.84% $\pm$ 0.33%
THDC-perfused rat livers				
1	0.193 $\pm$ 0.001	44.424% $\pm$ 3.29%	7.864% $\pm$ 0.92%	10.239% $\pm$ 1.52%
2	0.208 $\pm$ 0.004	44.796% $\pm$ 2.09%	8.177% $\pm$ 0.8%	8.507% $\pm$ 0.93%
8	0.444 $\pm$ 0.008	43.735% $\pm$ 0.98%	9.373% $\pm$ 1.76%	8.298% $\pm$ 1.35%
9	1.47 $\pm$ 0.16	44.94% $\pm$ 1.2%	8.07% $\pm$ 0.3%	8.23% $\pm$ 0.95%
10	1.17 $\pm$ 0.4	43.94% $\pm$ 2.8%	7.94% $\pm$ 0.52%	7.74% $\pm$ 1.7%

Phosphatidylcholine was isolated and separated by thin layer chromatography and then subjected to high-performance liquid chromatography (HPLC) for the sub-fractionation of the fatty acid composition as described in the methods section for control perfused livers and in livers perfused with taurohydoxychoic acid (THDC). Results are presented as mean  $\pm$  SE ( $n = 6$ ). No significant differences were found between the fatty acid composition of livers perfused with THDC when compared to the control; PC: Phosphatidylcholine.

enterohepatic circulation has little effect on biliary lipid composition and secretion. In contrast, Sinhal *et al.*<sup>[36]</sup> and Cohen *et al.*<sup>[37]</sup> showed that feeding THDC to hamsters and prairie dogs increased hepatic HMG-CoA reductase activity and thus an increase in vesicles carrying cholesterol. It is speculated that a similar increase in the activity

of PDE is caused in this experiment by THDC.

Analysis of the microsomal fraction gave an interesting profile of total phospholipids, cholesterol, protein and PDE, as presented in Figure 2. The results indicate that there may be two different populations of the parameters measured. The first population isolated from



**Figure 3** A typical high-performance liquid chromatography chromatogram of a liver homogenate. Phospholipids were separated by thin layer chromatography (TLC) and the phosphatidylcholine band was scraped off the TLC plate and extracted with 2 × 1 mL methanol [high-performance liquid chromatography (HPLC) grade]. After determination of the phospholipid, 20 nmol was injected onto HPLC for the analysis of the fatty acid composition of the phosphatidylcholine as described in the methods section. Results are presented as mean ± SE ( $n = 6$ ). A typical chromatogram is presented. Peak 1: Not identified; peak 2: 1-palmitoyl 2-arachidonoyl (16:0-20:4) phosphatidylcholine; peak 3: 1-palmitoyl 2-linoleoyl (16:0-18:2) phosphatidylcholine; peak 4: 1-palmitoyl 2-oleoyl (16:0-18:1) phosphatidylcholine; peak 5: 1-stearoyl 2-arachidonoyl (18:0-20:4) phosphatidylcholine.

fractions 1-2 ( $\rho = 1.05$ - $1.07$  g/mL) had relatively small amounts of cholesterol and PDE activity, whereas the population isolated from fractions 8-10 ( $\rho = 1.09$ - $1.23$  g/mL) had higher concentrations of phospholipids, cholesterol, protein and PDE activity (Figure 2A-D). Some putative vesicles may be present in fractions 8-10 ( $\rho = 1.09$ - $1.23$  g/mL) since these had more total phospholipids, cholesterol, protein and PDE activity. Enzyme activity in THDC-infused liver microsomal sub-fractions ( $\rho = 1.05$ - $1.07$  g/mL and  $\rho = 1.95$ - $1.23$  g/mL) was significantly higher than that observed in control values (Figure 2D). No significant difference was found in PC molecular species in the C16:0-C18:2, C16:0-C18:1 between control and THDC-infused liver subfractions.

In the experiments reported in this study, the isolation of biliary type vesicles was achieved by using the novel gradient medium, Iodixanol, which is a nonionic medium that has an advantage over sucrose in that it rapidly forms self-generated gradients in vertical or near-vertical rotors<sup>[38]</sup>. Increased lipid transfer vesicles might be present in the microsomal fraction<sup>[39,40]</sup>; however, subfractions of microsomal fraction on density gradient with Iodixanol failed to identify biliary transfer vesicles.

Crawford *et al.*<sup>[32,41]</sup> reported that vesicles are secreted from the outer leaflet of the canalicular membrane by ABCB4 transporter and subsequently, bile salt/phospholipid micelles in bile extract cholesterol from these vesicles. Vesicular secretion is compatible with the function of ABCG5/ABCG8, and several studies<sup>[7,32,42,43]</sup> have suggested that vesicular secretion of cholesterol is one of the mechanisms by which sterols appear in bile.

According to the results of this study no significant changes in biliary lipid-carrying vesicles were observed;

however, a significantly different profile of PDE was seen. However, the observation of increased vesicle accumulation during bile salt secretion by electron microscopy<sup>[44]</sup> and inhibition of the vesicle transport by colchicine, vinblastine and valproate still require explanation<sup>[45]</sup>. The changing of lipid content in any subcellular compartment might be prevented by analysis of the whole liver. However, the subcellular fraction of the livers which was also an initial fraction resulted in no significant difference between control and THDC-perfused rat livers (Figure 1A and B). Some putative biliary lipid transfer vesicles may exist but techniques used in this study have failed to identify them. The identification and regulation of biliary lipid-carrying vesicles may lead to an improvement in the treatment of hepatic disorders.

In conclusion, the present study failed to identify an increase in biliary lipid-carrying vesicles in THDC-infused rat livers probably due to the limitation of the techniques. However, PDE activity was significantly increased in the microsomal sub-fractions isolated from THDC-infused livers when compared to control values and needs further investigation.

## COMMENTS

### Background

Bile contains many constituents, including cholesterol and phospholipids, and these are reported to be transported from the hepatocytes to the bile canaliculus in vesicles. An increase in biliary lipid secretion can have implications for hepatic disorders such as cholesterol gallstone formation. Hence, the isolation of biliary lipid-carrying vesicles may lead to a better understanding of such hepatic disorders.

### Research frontiers

Taurohyodeoxycholic acid (THDC) is reported to increase biliary lipid-carrying vesicles (mainly phosphatidylcholine) and ultracentrifugation techniques are now available which can be used to separate vesicle type material relatively quickly.

### Innovations and breakthroughs

Biliary phosphatidylcholine has a unique fatty acid composition compared to hepatic phosphatidylcholine and can be identified by high-performance liquid chromatography (HPLC). Since THDC induces an increase in biliary phosphatidylcholine it was thought that increased biliary phosphatidylcholine carrier vesicles would be present in the hepatocytes and could be separated by ultracentrifugation. Electron microscopy has confirmed the presence of increased vesicles and lamellar bodies around and within the bile canaliculus after THDC infusion.

### Applications

Although the present study failed to identify an increase in biliary lipid-carrying vesicles in THDC-infused livers, probably due to the limitation of the techniques employed, vesicles enriched in phosphodiesterase I (PDE) activity were present in THDC livers compared to controls.

### Terminology

THDC is a natural 6 $\alpha$ -hydroxylated bile acid displaying hydrophilic properties and causes more secretion of phosphatidylcholine into bile compared to other bile acids. The microsomal fraction was obtained and subjected to further ultracentrifugation using a novel gradient centrifugation technique. The phosphatidylcholine was separated and subjected to HPLC fractionation since biliary phosphatidylcholine has a unique fatty acid composition.

### Peer review

In this study the authors have isolated rat livers and have subjected them to THDC infusion, which is reported to increase biliary phospholipid secretion. The livers were then homogenized and the microsomal fraction was subjected to ultracentrifugation by using a novel gradient technique in order to isolate putative vesicles destined for biliary secretion. The results show that the density

gradient fraction envisaged to contain the putative vesicle population isolated from THDC-perfused livers had relatively small amounts of phospholipids and protein when compared to the relevant control fractions. However, the vesicles isolated from the THDC-perfused livers displayed an increase in cholesterol and PDE activity.

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## Nilotinib-mediated mucosal healing in a rat model of colitis

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### Abstract

**AIM:** To investigate the effects of nilotinib in a rat model of trinitrobenzene sulfonic acid (TNBS)-induced colitis.

**METHODS:** Twenty-one Wistar albino female rats obtained from Dokuz Eylul University Department of Laboratory Animal Science were categorized into a control ( $n = 7$ ), TNBS ( $n = 7$ ) and nilotinib group ( $n = 7$ ). Saline was administered orally for 14 d to the control and the TNBS group. The TNBS group received rectal TNBS on the first day while saline was administered to the control group. The nilotinib group received 20 mg/kg nilotinib for 14 d in 2 divided doses, starting the

same day as TNBS administration. For 14 d, the rats were fed a standard diet, and their weights were recorded daily. After sacrifice, colon tissue samples from each group were scored for macroscopic and microscopic pathology. Apoptotic indices were determined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method. Platelet-derived growth factor receptor (PDGFR) alpha and beta levels were assessed through immunohistochemistry staining scores and compared among the groups. Tissue and serum tumor necrosis factor (TNF) alpha levels were determined by enzyme-linked immunosorbent assay.

**RESULTS:** Between days 1 and 14, the nilotinib group rats lost significantly less weight than the TNBS group rats ( $-0.7$  g *vs*  $-14.0$  g,  $P = 0.047$ ). The difference in weight between the control and nilotinib groups was also statistically significant ( $+8.3$  g *vs*  $-0.7$  g,  $P = 0.031$ ). From day 7 to day 14, the weight differences of the control group *vs* the TNBS group, the TNBS group *vs* the nilotinib group, and the control group *vs* the nilotinib group were all statistically significant ( $+8.0$  g *vs*  $-11.1$  g,  $P = 0.007$ ;  $-11.1$  g *vs*  $+2.9$  g,  $P = 0.015$ ;  $+8.0$  g *vs*  $+2.9$  g,  $P = 0.042$ , respectively). Macroscopic and microscopic scores were significantly lower in the nilotinib group than in the TNBS group ( $0.00 \pm 0.00$  *vs*  $1.43 \pm 0.65$ ,  $P = 0.009$ ;  $2.86 \pm 0.55$  *vs*  $7.71 \pm 1.48$ ,  $P = 0.030$ , respectively). However, these scores were similar between the nilotinib and control groups. While no significant difference for the nilotinib *vs* control groups could be determined for PDGFR alpha and beta scores, PDGFR alpha and beta scores were lower in the nilotinib group than in the TNBS group. Furthermore, the TNF alpha levels in the serum, tissue and apoptosis scores were similar between the nilotinib and TNBS groups.

**CONCLUSION:** Nilotinib prevents weight loss, facilitates mucosal healing by improving the pathological scores without introducing variation into the apoptotic scores or TNF alpha levels.

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**Key words:** Inflammatory bowel disease; Platelet-derived growth factor receptor; Tumor necrosis factor alpha; Tyrosine kinase inhibitor; Mucosal healing

**Core tip:** Unresponsiveness to medical treatment in refractory inflammatory bowel disease (IBD) still poses a therapeutic challenge. To detect an alternative treatment option, we selected nilotinib based on the fact that tyrosine kinases inhibitors affect several key components in the pathogenesis of IBD, including tumor necrosis factor (TNF) alpha, platelet-derived growth factor receptor (PDGFR), and apoptosis. In a trinitrobenzene sulfonic acid-induced colitis rat model, we concluded that nilotinib has a significant effect on weight loss and on macroscopic and microscopic pathological scores, leading to significant mucosal healing. Although nilotinib caused a decrease in the PDGFR alpha and PDGFR beta levels, it did not have a significant effect on the apoptotic scores or TNF alpha levels.

Ataca P, Soy Turk M, Karaman M, Unlu M, Sagol O, Dervis Hakim G, Yilmaz O. Nilotinib-mediated mucosal healing in a rat model of colitis. *World J Gastroenterol* 2013; 19(37): 6237-6244 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6237.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6237>

## INTRODUCTION

Chronic intestinal inflammation is characterized by the pathological responses of the adaptive and innate immune systems. These responses are central to the pathological mechanisms that lead to inflammatory bowel disease (IBD)<sup>[1]</sup>. Genetic and environmental factors, infectious agents, the structure of the enteric flora, and immune system dysfunctions are key elements in the pathogenesis of IBD, and thus, these are targets for many drugs developed to treat IBD<sup>[2,3]</sup>. However, unresponsiveness to medical treatment in IBD still poses a therapeutic challenge. Previous studies examining the therapeutic effectiveness of selecting drugs in patients with ulcerative colitis (UC) reported the rates of remission to be 47%-81% with rectal 5-aminosalicylic acid (5-ASA), 9%-30% with oral 5-ASA, and 42%-82% with thiopurines<sup>[4-6]</sup>.

Monoclonal tumor necrosis factor (TNF) alpha inhibitors are currently the treatment of choice, especially in severe and resistant cases of IBD. However, decreased responses or resistance to the TNF alpha inhibitor infliximab have been reported. Previous studies have reported an average clinical remission rate at week 8 of 33% (range, 27.5%-38.8%) with the use of infliximab in IBD patients<sup>[7]</sup>. Clinical remission was maintained in 33% (range, 25.6%-36.9%) of patients treated with infliximab at week 30<sup>[7]</sup>. In a randomized, placebo-controlled 52-wk study examining the effectiveness of adalimumab, another anti-TNF agent, the IBD remission rate was significantly

higher than the placebo, regardless of treatment with steroids (13.3% and 5.7%, respectively;  $P = 0.035$ )<sup>[8]</sup>.

Mucosal healing has emerged as a key therapeutic objective in the treatment of IBD and is able to predict sustained clinical remission and resection-free survival in patients. Mucosal healing is achieved in approximately 30% of IBD patients receiving corticosteroid therapy and in as many as 60% of IBD patients receiving anti-TNF therapies<sup>[9-11]</sup>. Approximately 20% of IBD patients, however, do not respond to anti-TNF therapy and require surgical intervention<sup>[12]</sup>. These findings emphasize the importance of discovering new medical treatment options for IBD because the currently available treatments are insufficient for a substantial number of patients.

Tyrosine kinases (TKs) are enzymes that play a role in normal cell function, metabolism, growth, differentiation, and apoptosis. TK inhibitors are drugs that block the action of these enzymes. Although they are typically used as anticancer drugs, they have recently been considered for use in noncancer proliferative diseases and for inflammatory conditions. Imatinib, the best-known member of this class of drugs, is specific for TK receptor sites and suppresses the Abelson proto-oncogene (ABL), the c-kit proto-oncogene, platelet-derived growth factor receptor (PDGFR), macrophage colony-stimulating factor receptor, TNF alpha, and inducible nitric oxide synthase<sup>[13]</sup>. Nilotinib is a more potent inhibitor of TKs than imatinib. In studies involving patients with lung fibrosis, nilotinib has been shown to reduce interleukin (IL)-6, IL-1 beta, TNF alpha, tumor growth factor beta 1, and PDGFR beta levels more significantly than imatinib and had a potent antifibrotic effect<sup>[14]</sup>.

In the literature, there are a few reports suggesting that TK inhibitors may be effective in IBD. In a case report by Magro *et al.*<sup>[15]</sup>, a patient diagnosed with Crohn's disease (CD) and chronic myeloid leukemia (CML) remained in remission for 3 years on imatinib therapy alone, without the use of mesalamine or steroids. Cuzzocrea *et al.*<sup>[16]</sup> demonstrated that the development of colitis in dinitrobenzene sulfonic acid (DNBS)-induced colitis animal models was reduced by the TK inhibitor tyrphostin AG126.

The present study was planned based on the demonstrated success of nilotinib in previous studies and on the fact that TK inhibitors affect several key components in the pathogenesis of IBD, including TNF alpha, PDGFR, and nitric oxide (NO) synthesis. For this purpose, we evaluated the efficacy of nilotinib on weight, macroscopic and microscopic pathological scores, TNF alpha levels, PDGFR levels, and the apoptotic index in a rat model of trinitrobenzene sulfonic acid (TNBS)-induced colitis. This study is the first to evaluate the efficacy of nilotinib in a rat colitis model.

## MATERIALS AND METHODS

### Experimental design

Approval was obtained from the animal ethics council of Dokuz Eylul University Medical Faculty (DEUTF). The

DEUTF Hospital Experimental Research Laboratory provided 21 female Wistar albino rats weighing 200-250 g (mean weight:  $209.43 \pm 8.92$  g) for use in this study.

The rats were maintained in a room at a temperature of  $23 \pm 2$  °C under a 12-h light/dark cycle at the DEUTF Experimental Animal Laboratory. Before and during the study, they were fed a standard diet, and their weights were monitored daily. The animals were also allowed water *ad libitum*.

The rats were divided into 3 groups, each consisting of 7 rats: the control group, TNBS group and nilotinib group. After 24 h of fasting, 0.25 mL of the physiological serum was intracolonic administered to the control group rats through a cannula inserted 8 cm proximal to the anus, using a rectally inserted flexible polypropylene catheter. To induce colitis, the rats in the other 2 groups received an intracolonic solution treated with 0.5 mL of 100 mg/mL TNBS (Sigma, Germany) dissolved in 30% ethanol and administered through a cannula. Before catheter insertion, short-term sedation was provided through ether anesthesia. Neither group of rats treated with TNBS encounter any instance of perforation or death due to colonic ulceration. The TNBS and control groups received a saline placebo for 14 d through an orogastric tube. Nilotinib 20 mg/kg/d (Novartis Pharma AG, Basel, Switzerland) was administered in 2 divided doses to the nilotinib group for 14 d through an orogastric tube, beginning on the same day as TNBS administration.

Blood and tissue samples for pathological examination were obtained from all of the rats under ether anesthesia at the end of the 14-d period. All of the animals were then sacrificed by decapitation. The abdominal cavities were opened by a midline incision, and the entire length of the large intestines was dissected from the distal ileum to the rectum. After washing with saline, the large intestinal tissues were fixed with buffered formalin.

### Pathological examination

A pathologist blinded to the group identity of the intestinal samples performed pathological evaluations of all of the tissue samples. Each intestinal column was opened longitudinally, according to the method reported by Vilaseca *et al.*<sup>17</sup>, and macroscopic scoring was performed. Tissue sections of the gross ulcerative lesions and surrounding normal mucosa were then stained with hematoxylin-eosin (HE). The pathologist then performed microscopic scoring according to the method reported by Dieleman *et al.*<sup>18</sup>.

### Apoptosis

The pathologist stained all tissue samples using the TUNEL method. Mucosal crypts and apoptotic cells were counted along the surface epithelium under a microscope (Olympus DX51) at a magnification of  $\times 400$ . Using the TUNEL technique, all of the cut sections were preserved with lysine for 3 nights at 37 °C and then for 1 night at 60 °C in an incubator. Thereafter, deparaffinization was performed with 3 cycles of xylene. The tissue sections

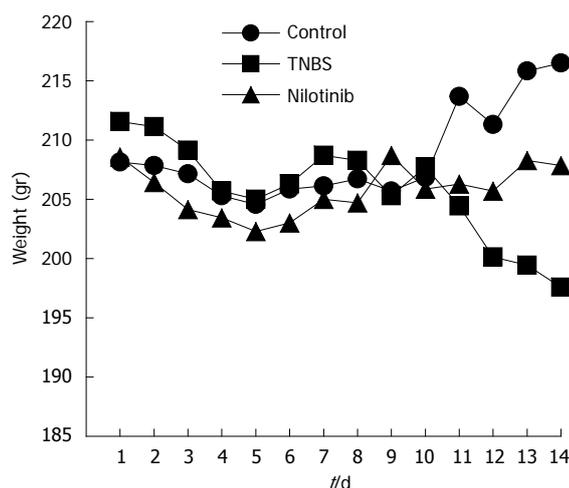
were then rehydrated by flushing with a series of alcohol solutions of decreasing degrees (absolute, 96%, 80%, and 70%) and then stored in distilled water for 5 min. Proteinase K (Proteinase K, Invitrogen, Carlsbad, CA, United States) was applied for 10 min at room temperature. The sections were then washed twice with phosphate-buffered solution (PBS) for a period of 2 min each. After drying the cross-sections, 3% H<sub>2</sub>O<sub>2</sub> (Merck, Germany) was applied for 5 min, and the sections were then washed with PBS twice for 5 min each. The cross-sectional slices were then dried, and an equilibration buffer (ApopTag Plus peroxidase kit, Millipore, Billerica, MA, United States) was applied for 10 min at room temperature. A total of 55  $\mu$ L of the enzyme terminal deoxynucleotidyl transferase was then applied to each cross-section. The cross-sections were closed with a coverslip (ApopTag Plus peroxidase kit, Millipore, Billerica, MA, United States) and incubated for 1 h at 37 °C. Stop/wash buffer (ApopTag Plus peroxidase kit, Millipore, Billerica, MA, United States) was then applied to the sections removed from the incubator for 10 min at room temperature. The sections were then washed with PBS at room temperature 3 times for 1 min each, dried, and incubated with anti-streptavidin-peroxidase (ApopTag Plus peroxidase kit, Millipore, Billerica, MA, United States) at room temperature for 30 min. The sections were then washed with PBS 4 times for 2 min to determine the visibility of the TUNEL reaction before being stained with diaminobenzidine (DAB) (DAB-PLUS kit; Invitrogen, Carlsbad, CA, United States). After washing with distilled water, ground staining was performed using methyl green. After three changes of the searing process with xylene for 20 min, it was closed with Entella.

### Tissue homogenization and measurement of tissue serum TNF alpha

The tissue samples obtained from the ileum were introduced into 2 mL microcentrifuge tubes and stored at -80° C until the day of the study. These tissues were then removed and warmed to 4 °C. Next, 60-80 mg pieces were obtained from these samples and placed into a tube containing 5-mm-diameter stainless steel beads and a phosphate buffer with a 1:7 ratio (pH 7.2). Microcentrifuge tubes were introduced into a pre-chilled TissueLyser LT device and replaced into a TissueLyser (Qiagen-Germany) tissue homogenization device. Next, an enzyme-linked immunosorbent assay (ELISA) was performed on tissue supernatants, and serum was obtained via centrifugation for the identification of TNF alpha in accordance with the manufacturer's recommendations (Invitrogen Rat TNF-alpha, Carlsbad, CA, United States). Finally, the ELISA plates were spectrophotometrically evaluated at 450 nm (Biotech Synergy HT; Winooski, VT, United States).

### PDGFR alpha and beta levels

PDGFR alpha and beta levels were assessed through staining scores and compared among the groups by im-



**Figure 1** Trends of weight changes among the experimental groups. Control group (circle), Trinitrobenzene sulfonic acid (TNBS) group (square), and nilotinib group (triangle). The TNBS group lost an average weight of 14 g, while the nilotinib group lost 0.7 g in 14 d ( $P = 0.047$ ). The nilotinib group gained an average weight of 2.9 g between day 7 and day 14, while the TNBS group lost an average weight of 11.1 g ( $P = 0.015$ ).

munohistochemistry. For immunohistochemical staining, 2-3 micron sections were stored overnight in an incubator at 40 °C. The following day, the sections were washed with xylene, a descending alcohol series, and distilled water for 20 min. They were then boiled for 20 min in EDTA solution at pH 8. Next, they were stored in DakoFlex peroxidase solution for 5 min and washed again with Tris-buffered saline. A primary antibody was then applied. PDGFR alpha in a 1:100 dilution (NOVUS Biologicals, NBP1-19 423, Littleton, CO, United States) and PDGFR beta in a 1:50 dilution (NOVUS Biologicals, NBP1-19 473; Littleton, CO, United States) were stored for 30 min, washed with Tris buffer, stored in DakoFlex HRP solution for 20 min, washed with Tris buffer again, and stored in DakoFlex DAB for 7 min. The samples were again washed with Tris-buffered saline, kept under tap water for 5 min, stained with Mayer's hematoxylin solution for 10 min, washed with tap water for 1 min, rinsed in an alcohol series, and cleaned with xylene for 5-10 min.

PDGFR alpha and beta positivity was determined according to a devised scoring system. According to this system, a score of 1 was assigned if PDGFR alpha and beta positivity was confirmed in inflammatory cells and in the cells of the lamina propria, stroma, and submucosal endothelium. A score of 2 was assigned if increased expression of PDGFR alpha and beta was confirmed in the lamina propria and submucosa. A score of 3 was assigned if PDGFR alpha and beta positivity was confirmed with widespread staining in the ulcerated areas or in the inflammatory cells, fibroblasts, endothelial cells, submucosa, and mucosa of the surrounding tissue.

### Statistical analysis

All statistical procedures were performed using SPSS software (version 15.0). The Kruskal-Wallis test was used

for multigroup comparisons, and the Mann-Whitney *U* test was used to compare the means of 2 groups. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

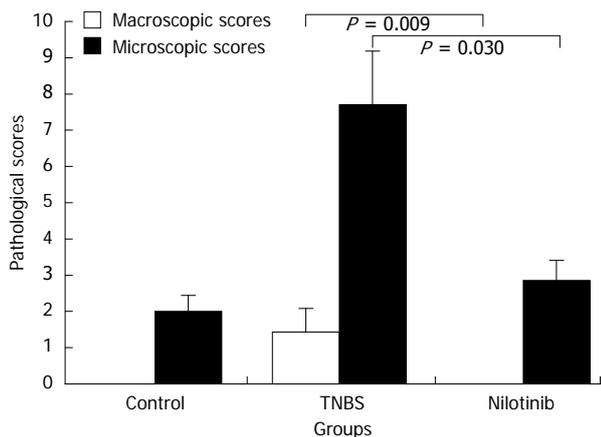
Bloody diarrhea was observed on day 1 of rectal TNBS administration in all 14 of the rats in the 2 experimental groups; no bloody diarrhea was observed in the control group. In the TNBS and nilotinib groups, the diarrhea was semi-solid on day 5. In the nilotinib group, normal stools were observed after day 7. During rectal saline administration under ether anesthesia in the control group, respiratory arrest developed in 1 rat, which remained stable after CPR. However, the animal's general condition deteriorated over the next few d, and the animal died on day 6 of the experiment. An autopsy was not performed on this rat.

On the first experimental day, the average weights were similar among all of the study groups ( $P > 0.05$ ), and the average weights were examined daily (Figure 1). The average weight of the control group increased to 8.3 g at the end of 14 d. The TNBS group, however, lost an average of 14 g throughout the study, and the nilotinib group lost an average of 0.7 g. There was a significant difference among the groups with regard to the average weight change throughout the study ( $P = 0.006$ ). The difference in weight between the control and nilotinib groups was statistically significant (+8.3 and -0.7 g, respectively,  $P = 0.031$ ). The TNBS group lost significantly more weight than the nilotinib group (-14.0 and -0.7 g, respectively;  $P = 0.047$ ) and the control group (-14.0 and +8.3 g, respectively,  $P = 0.008$ ).

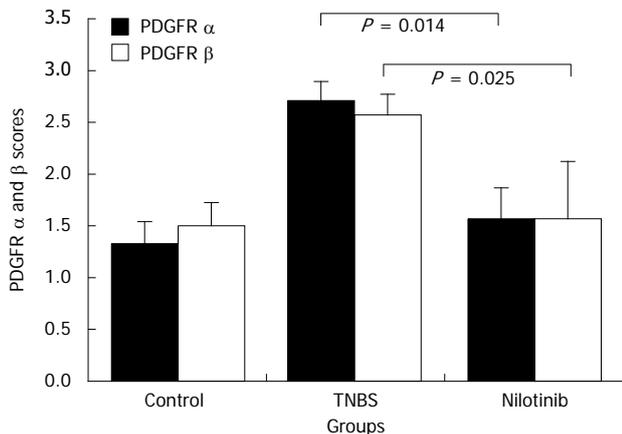
Between day 7 and day 14, the weights of the control group increased by an average of 8 g; those of the nilotinib group increased by an average of 2.9 g; and those of the TNBS group decreased by an average of 11.1 g. Comparing the average increase in weights over this time period among all 3 of the groups, there was a significant difference observed ( $P = 0.004$ ). From day 7 to day 14, the weight differences of the control rats *vs* the TNBS rats, the TNBS rats *vs* the nilotinib rats, and the control rats *vs* the nilotinib rats were statistically significant (+8.0 and -11.1 g,  $P = 0.007$ ; -11.1 and +2.9 g,  $P = 0.015$ ; +8.0 and +2.9 g,  $P = 0.042$ , respectively).

The mean macroscopic pathological scores of the control and nilotinib groups were 0, while the macroscopic pathological score in the TNBS group was  $1.43 \pm 0.65$ . When the distribution of macroscopic scores based on rats was examined, all scores from the control and nilotinib group rats were "0", which is noteworthy. The control and nilotinib groups were similar in terms of macroscopic scores ( $P > 0.05$ ). Macroscopic scores were significantly lower in the control and nilotinib groups than in the TNBS group ( $0.00 \pm 0.00$  and  $1.43 \pm 0.65$ ,  $P = 0.014$ ;  $0.00$  and  $1.43 \pm 0.65$ ,  $P = 0.009$ , respectively) (Figure 2).

The mean microscopic scores in the control, TNBS, and nilotinib groups were  $2.0 \pm 0.45$ ,  $7.71 \pm 1.48$ , and  $2.86 \pm 0.55$ , respectively. The mean microscopic scores were significantly lower in the control and nilotinib groups than



**Figure 2** Microscopic and macroscopic pathological scores among the experimental groups. The results are the mean ± SD. Macroscopic and microscopic pathological scores were similar in the control and nilotinib groups, while the scores in the nilotinib group were significantly lower than those in the trinitrobenzene sulfonic acid (TNBS) group (TNBS vs nilotinib,  $P = 0.009$ ; TNBS vs nilotinib,  $P = 0.030$ ).

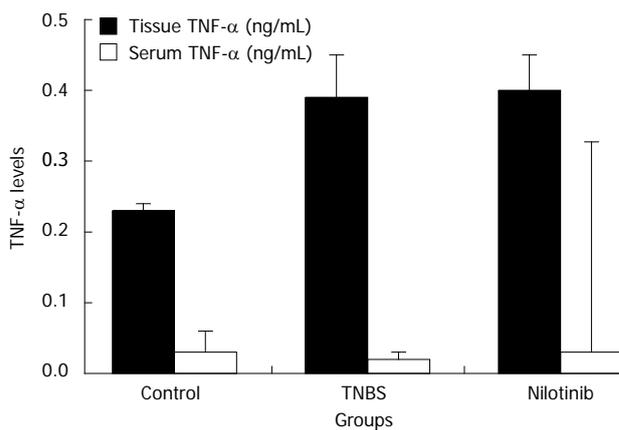


**Figure 3** Platelet-derived growth factor receptor alpha and beta scores among the experimental groups. The results are the mean ± SD. Platelet-derived growth factor receptor (PDGFR) alpha and beta scores were similar in the control and nilotinib groups, while the scores in the nilotinib group were significantly lower than those in the trinitrobenzene sulfonic acid (TNBS) group (TNBS vs nilotinib,  $P = 0.014$ ; TNBS vs nilotinib,  $P = 0.025$ ).

in the TNBS group ( $2.0 \pm 0.45$  and  $7.71 \pm 1.48$ ,  $P = 0.034$ ;  $2.86 \pm 0.55$  and  $7.71 \pm 1.48$ ,  $P = 0.030$ , respectively). The control and nilotinib groups were similar in terms of the mean microscopic scores ( $P > 0.05$ ) (Figure 2).

With regard to the PDGFR alpha and beta scoring system, the PDGFR alpha scores in the control, TNBS, and nilotinib groups were  $1.33 \pm 0.21$ ,  $2.71 \pm 0.18$ , and  $1.57 \pm 0.30$ , respectively. There was a significant difference among the groups ( $P = 0.007$ ). The PDGFR alpha scores were significantly lower in the control and nilotinib groups than in the TNBS group ( $1.33 \pm 0.21$  and  $2.71 \pm 0.18$ ,  $P = 0.004$ ;  $1.57 \pm 0.30$  and  $2.71 \pm 0.18$ ,  $P = 0.014$ , respectively). The control and nilotinib groups were similar in terms of the PDGFR alpha scores ( $P > 0.05$ ) (Figure 3).

The mean PDGFR beta scores in the control, TNBS,



**Figure 4** Tissue and serum tumor necrosis factor α levels among the experimental groups. The results are the mean ± SD. Serum tumor necrosis factor (TNF) and tissue TNF α levels were similar between the trinitrobenzene sulfonic acid (TNBS) and nilotinib groups.

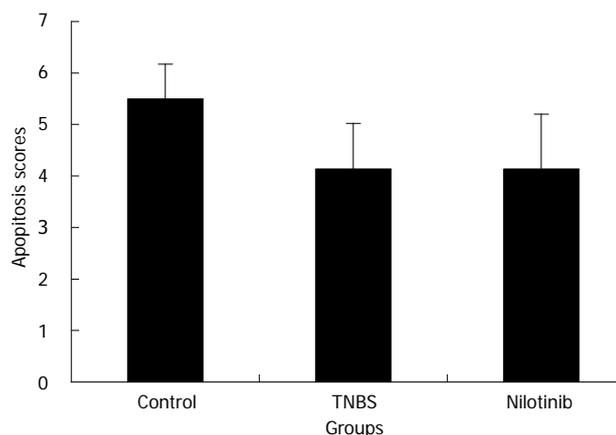
and nilotinib groups were  $1.50 \pm 0.22$ ,  $2.57 \pm 0.20$ , and  $1.57 \pm 0.30$ , respectively. There was a statistically significant difference among all of the groups in terms of the mean PDGFR beta scores ( $P = 0.020$ ). The PDGFR beta scores were significantly lower in the control and nilotinib groups than in the TNBS group ( $1.50 \pm 0.22$  and  $2.57 \pm 0.20$ ,  $P = 0.011$ ;  $1.57 \pm 0.30$  and  $2.57 \pm 0.20$ ,  $P = 0.025$ , respectively). The PDGFR beta scores of the control and nilotinib groups were similar ( $P > 0.05$ ) (Figure 3).

The mean serum TNF alpha levels in the control, TNBS, and nilotinib groups were  $0.03 \pm 0.03$ ,  $0.02 \pm 0.01$ , and  $0.03 \pm 0.01$  pg/mL, respectively. There was no statistically significant difference observed among the groups in terms of the mean serum TNF alpha levels ( $P > 0.05$ ) (Figure 4). The average tissue TNF alpha levels in the control, TNBS, and nilotinib groups were  $0.23 \pm 0.01$ ,  $0.39 \pm 0.06$ , and  $0.40 \pm 0.05$  ng/mL, respectively. There was a significant difference observed among the groups ( $P = 0.002$ ). TNF alpha levels were significantly lower in the control group than in the TNBS or nilotinib groups ( $0.23 \pm 0.01$  and  $0.39 \pm 0.06$  ng/mL,  $P = 0.002$ ;  $0.23 \pm 0.01$  and  $0.40 \pm 0.05$  ng/mL,  $P = 0.003$ , respectively). However, there was no statistically significant difference between the TNBS and nilotinib groups in terms of the mean tissue TNF alpha levels ( $P > 0.05$ ) (Figure 4).

The mean number of apoptotic cells detected by the TUNEL method in the control, TNBS, and nilotinib groups was  $5.50 \pm 0.67$ ,  $4.14 \pm 0.88$ , and  $4.14 \pm 1.06$ , respectively. The difference among the groups was not statistically significant ( $P > 0.05$ ) (Figure 5).

## DISCUSSION

IBDs, such as CD and UC, are chronic recurrent intestinal inflammatory conditions. Genetic, environmental, microbial, and immune factors play a role in the etio-pathogenesis of IBDs. Despite the development of biological therapies and advancements in genetic technology, treatment options remain limited for refractory cases.



**Figure 5 Apoptosis scores among the experimental groups.** The results are the mean  $\pm$  SD. Apoptosis scores were similar among the groups. TNBS: Trinitrobenzene sulfonic acid.

Mucosal healing has emerged as a key treatment goal for IBD and allows the prediction of sustained clinical remission and resection-free survival in affected patients. Mucosal healing can be achieved in approximately 30% of patients receiving corticosteroid therapy and in 60% of patients receiving anti-TNF agents<sup>[9-12]</sup>. Approximately 20% of IBD patients do not respond to anti-TNF therapy and require surgical intervention<sup>[12]</sup>. Thus, the currently available medical treatment options are ineffective in a substantial group of patients with IBD.

Nilotinib, which was used in this study, is a strong TK inhibitor that was initially approved for use in patients with imatinib-intolerant and imatinib-resistant Philadelphia chromosome-positive chronic or accelerated phase-CML and has since been approved as a frontline therapy in chronic phase CML<sup>[19]</sup>. Nilotinib is more potent than imatinib, which inhibits the autophosphorylation of various kinases, such as BCR-ABL, PDGFR, and c-KIT<sup>[19]</sup>. Nilotinib is generally well tolerated. Due to the lack of Src family kinase inhibition, myelosuppression is an infrequent adverse event that occurs less frequently with nilotinib than with other TK inhibitors<sup>[19]</sup>. The most common manageable adverse events are rash, pruritus, fatigue and headache. Neutropenia, anemia, thrombocytopenia, elevations of liver enzymes, cardiac toxicity, namely QT prolongation, fluid retention, edema, and weight gain are among the less common side effects<sup>[19]</sup>. TK inhibitors affect several key components in the pathogenesis of IBD, including TNF alpha, PDGFR, and NO synthesis. In this study, we evaluated the efficacy of nilotinib on weight, macroscopic and microscopic pathological scores, TNF alpha and PDGFR levels, and the apoptotic index in rat models with TNBS-induced colitis. There are no previous reports in the literature evaluating the efficacy of nilotinib in either a rat model of colitis or in human colitis.

In the present study, the weights of the control and experimental rats were monitored daily. At the end of 14 d, rats in the nilotinib group had lost significantly less weight than rats in the TNBS group ( $P = 0.047$ ). These results are similar to those obtained in the study by Cuzzocrea *et al*<sup>[16]</sup>, in which weight loss was significantly

reduced by 7 d of treatment with the TK inhibitor tyrophostin AG126 in a DNBS-induced colitis animal model. The TK inhibitor used in the study by Cuzzocrea *et al*<sup>[16]</sup> is different from that used in our study. However, our study indicates that nilotinib does have a positive effect on weight in animal models with colitis.

The first therapeutic target in drug studies for the treatment of IBD was the regression of disease-related symptoms. The most important reason for this was that the agents used in the treatment of IBD were not disease-modifying drugs. In more recent studies, however, the primary endpoint in evaluating the therapeutic efficacy of drugs used to treat IBD has been “mucosal healing”<sup>[20]</sup>. With mucosal healing as the therapeutic target, continuous clinical remission and survival without surgery can be achieved<sup>[21]</sup>. The mucosal healing rates of anti-TNF agents have been reported at approximately 60% in the active ulcerative colitis trials (ACT)-1 and ACT-2 studies<sup>[7,10]</sup>. In the present study, the effects of nilotinib on mucosal healing and pathological macroscopic and microscopic scores yielded quite remarkable results. The macroscopic and microscopic pathological scores of intestinal tissue from the nilotinib group were similar to those of the control group ( $P > 0.05$ ) but significantly lower than those of the TNBS group ( $P = 0.009$ ;  $P = 0.030$ , respectively). In our study, the similar microscopic and macroscopic scores of the nilotinib and control groups constituted the most important evidence of the mucosal healing effect of nilotinib. Indeed, in the study conducted by Cuzzocrea *et al*<sup>[16]</sup>, the rats treated with the TK inhibitor showed significant histological improvements after treatment compared with the control rats. This result parallels the results of our study. Although there are no human studies investigating the use of TK inhibitors in patients with IBD, the case report by Magro *et al*<sup>[15]</sup> representing a case of long-standing remission from Crohn’s disease under imatinib therapy supports these results. However, a number of caveats should be noted regarding the present study. This is the first study of nilotinib in a rat colitis model. The current study was unable to compare the efficacy of nilotinib with that of other IBD agents or to assess the adverse events of nilotinib. Generally, as found in previous studies on CML, nilotinib has been a well-tolerated agent with manageable adverse effects. The findings of this study have a number of important implications for future practice. Further experimental investigations could provide more definitive evidence for human studies.

In our study, to determine the effectiveness of nilotinib on colitis, the PDGFR alpha, PDGFR beta, TNF alpha, and apoptosis levels were compared among the groups. Similar to the results observed in the macroscopic and microscopic pathological scores, the PDGFR alpha and beta scores were significantly lower in the nilotinib group than in the TNBS group ( $P = 0.014$ ,  $P = 0.025$ ) but were similar to the control group. There are no other studies investigating the effects of TK inhibitors on the levels of PDGFR alpha and beta in a colitis animal model. Histologically, in IBD, the intestinal microvascula-

ture shifts into a tight angiogenic structure characterized by the increased secretion of angiogenic integrins and mediators into the inflamed mucosa<sup>[22]</sup>. PDGF alpha and beta are 2 of the angiogenic mediators whose levels increase in IBD<sup>[23]</sup>. Increased PDGF alpha and beta activity can be found in the fibrotic areas adjacent to the active ulcer areas in IBD. Kumagai *et al.*<sup>[24]</sup> detected the increased expression of PDGF and PDGFR in the areas with active fibrosis in IBD, and they considered that this contributes to the development of IBD. The macrovascular results of this process were demonstrated as endothelial dysfunction in study of Principi *et al.*<sup>[25]</sup> due to decreased brachial artery flow-mediated vasodilatation in patients with IBD. The results of our study suggest that nilotinib enacts its effect on mucosal healing in colitis by blocking PDGFR alpha and beta.

TNF alpha is a protein that plays a role in cell proliferation, differentiation, and cell survival. It is responsible for the expression of adhesion molecules, fibroblast proliferation, the release of procoagulant substances, the initiation of cytotoxic apoptosis, and the acute phase response<sup>[26]</sup>. It has a clearly defined role in the pathogenesis of IBD, and anti-TNF agents are currently being used in the successful treatment of IBD<sup>[10]</sup>. In IBD, induced apoptosis can be triggered by TNF alpha, which causes much larger leaks in the intestinal barrier<sup>[27]</sup>. Previous studies have demonstrated that TNF alpha and IL-1 beta, both proinflammatory cytokines synthesized in the colon, are reduced with TK inhibition<sup>[16,28]</sup>. In our study, the apoptotic indices and serum and tissue levels of TNF alpha were evaluated. The serum and tissue levels of TNF alpha and the apoptotic index in the nilotinib group were found to be similar to those in the TNBS group. Previously, it has been shown that TNF alpha levels on day 7 are significantly higher in acute models of colitis established through a single dose application of TNBS, compared to the model of chronic colitis using weekly TNBS administrations<sup>[29,30]</sup>. That the serum and tissue TNF alpha levels were similar in the nilotinib and TNBS groups in our study might be explained by the length of the experiment (14 d), during which a TNF alpha peak could not be obtained. Additionally, the apoptosis indices were similar between both groups in our study. D'Argenio *et al.*<sup>[31]</sup> demonstrated the apoptotic cells and expressions of apoptotic proteins in TNBS-induced colitis over 4 wk. According to the results of this study, the apoptotic cell count was detected to be significantly decreased after first week by the TUNEL method<sup>[31]</sup>. The similar apoptotic scores detected in our study might be because the apoptotic cell peak could not be obtained after 14 d. Furthermore, the similar results of the TNF alpha levels and apoptosis scores in our study might also suggest that nilotinib has no significant effect on TNF alpha levels and apoptosis.

In conclusion, nilotinib has a significant effect on weight loss, as well as on the macroscopic and microscopic pathological scores in rats with TNBS-induced colitis, leading to significant mucosal healing. Although nilotinib caused a decrease in PDGFR alpha and PDGFR

beta levels, it did not have a significant effect on apoptotic scores or TNF alpha levels.

## COMMENTS

### Background

Genetic and environmental factors, infectious agents, the structure of enteric flora, and immune system dysfunction are key elements in the pathogenesis of inflammatory bowel disease (IBD); thus, these are the targets for many drugs developed to treat IBD. Unresponsiveness to medical treatments in refractory IBD still poses a therapeutic challenge. Tyrosine kinases (TKs) are enzymes that play a role in normal cell function, metabolism, growth, differentiation, and apoptosis. To establish an alternative treatment option, they selected nilotinib based on the fact that TK inhibitors affect several key components in the pathogenesis of IBD, including tumor necrosis factor (TNF) alpha, platelet-derived growth factor receptor (PDGFR), and apoptosis.

### Research frontiers

Nilotinib is a TK inhibitor that is typically used as an anticancer drug. Recently, it has been considered for use in noncancerous proliferative diseases and for inflammatory conditions. Authors concluded that nilotinib has a significant effect on weight loss and macroscopic and microscopic pathological scores while leading to significant mucosal healing. Although nilotinib caused a decrease in the PDGFR alpha and PDGFR beta levels, it did not have a significant effect on apoptotic scores or TNF alpha levels.

### Innovations and breakthroughs

Genetic, environmental, microbial, and immune factors play a role in the etio-pathogenesis of IBDs. The currently available medical treatment options are ineffective in a substantial group of patients with IBD. Nilotinib, as used in this study, is a strong TK inhibitor. TK inhibitors affect several key components in the pathogenesis of IBD, including TNF alpha, PDGFR, and nitric oxide synthesis. Before this study, there were no reports in the literature evaluating the efficacy of nilotinib in a rat colitis model. One previous study showed that the use of a different TK inhibitor could successfully treat rat colitis. There are no human studies investigating the use of TK inhibitors in patients with IBD.

### Applications

The results of this study suggest that nilotinib has a significant effect on weight loss, as well as on the macroscopic and microscopic pathological scores of rats with Trinitrobenzene sulfonic acid (TNBS)-induced colitis. Additionally, this treatment leads to significant mucosal healing and caused a decrease in PDGFR alpha and PDGFR beta levels, although it did not have a significant effect on apoptotic scores or TNF alpha levels. These results suggest that *nilotinib* may be effective in patients with IBD. The findings of this study have a number of important implications for the future practice. Therefore, further studies are needed to draw firm conclusions.

### Terminology

IBD are chronic inflammatory disorders of the gastrointestinal tract that have characteristic clinical, pathological, endoscopic and radiological features. TKs are enzymes that play a role in normal cell function, metabolism, growth, differentiation, and apoptosis. TK inhibitors are drugs that block the action of these enzymes. TNBS-induced colitis is a well-established animal model of mucosal inflammation that has been used for over 2 decades in the study of IBD pathogenesis, as well as in preclinical studies.

### Peer review

This is an excellent basic research animal study using a well-known model of TNBS-induced colitis in rats. The authors explored the ability of a tyrosine kinase inhibitor, nilotinib, to treat various clinical (weight determination), laboratory (TNF levels, apoptotic index) and pathological parameters (macroscopic and microscopic pathologic scores, PDGFR levels) and to quantify results. They were basically able to demonstrate mucosal healing effects, clinical improvements and decreased PDGFR alpha and beta levels; however, significant drops in the TNF peaks and apoptotic indices were not clearly shown.

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## Lymph node metastasis in gastric cardiac adenocarcinoma in male patients

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with lymphadenectomy in the Department of Surgery, Xin Hua Hospital and Rui Jin Hospital of Shanghai Jiaotong University Medical School between November 2001 and May 2012. Both the surgical procedure and extent of lymph node dissection were based on the recommendations of Japanese gastric cancer treatment guidelines. Univariate and multivariate analyses of lymph node metastases and the clinicopathological features were undertaken.

**RESULTS:** The rate of lymph node metastases in male patients with gastric cardiac adenocarcinoma was 72.1%. Univariate analysis showed an obvious correlation between lymph node metastases and tumor size, gross appearance, differentiation, pathological tumor depth, and lymphatic invasion in male patients. Multivariate logistic regression analysis revealed that tumor differentiation and pathological tumor depth were the independent risk factors for lymph node metastases in male patients. There was an obvious relationship between lymph node metastases and tumor size, gross appearance, differentiation, pathological tumor depth, lymphatic invasion at pN<sub>1</sub> and pN<sub>2</sub>, and nerve invasion at pN<sub>3</sub> in male patients. There were no significant differences in clinicopathological features or lymph node metastases between female and male patients.

**CONCLUSION:** Tumor differentiation and tumor depth were risk factors for lymph node metastases in male patients with gastric cardiac adenocarcinoma and should be considered when choosing surgery.

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**Key words:** Gastric neoplasm; Lymph node metastasis; Risk factors; Gastrectomy; Lymphadenectomy

**Core tip:** There is an obvious correlation between lymph node metastases and tumor size, gross appearance, differentiation, pathological tumor depth and lymphatic invasion in male patients. Tumor differentiation and

### Abstract

**AIM:** To reveal the clinicopathological features and risk factors for lymph node metastases in gastric cardiac adenocarcinoma of male patients.

**METHODS:** We retrospective reviewed a total of 146 male and female patients with gastric cardiac adenocarcinoma who had undergone curative gastrectomy

pathological tumor depth were independent risk factors for lymph node metastases in male patients. There was an obvious relationship between lymph node metastases and tumor size, gross appearance, differentiation, pathological tumor depth, lymphatic invasion at pN<sub>1</sub> and pN<sub>2</sub>, and nerve invasion at pN<sub>3</sub> in male patients. There were no significant differences in clinicopathological features or lymph node metastases between female and male patients.

Ren G, Chen YW, Cai R, Zhang WJ, Wu XR, Jin YN. Lymph node metastasis in gastric cardiac adenocarcinoma in male patients. *World J Gastroenterol* 2013; 19(37): 6245-6257 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6245.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6245>

## INTRODUCTION

Although its incidence and mortality have declined over the past 50 years, gastric cancer (GC) remains the fourth most common cancer and the second most frequent cause of cancer death worldwide<sup>[1-3]</sup>. In China, GC is the second most common malignancy and the third most frequent cause of cancer-related death, with an annual age-adjusted mortality rate of 24.34 deaths per 100000 people<sup>[4]</sup>. As GC incidence declines, the frequency of proximal gastric and gastroesophageal junctional adenocarcinomas continues to rise, and has become a significant clinical challenge<sup>[5-7]</sup>. The reasons for this rapid increase in aggressive proximal malignancies remain unclear. Tumors in the upper third of the stomach might spread *via* the lymphatic system through the lower esophageal channel to the mediastinum, through the suprapancreatic channel to the abdomen, or through the abdominal para-aortic channel to the retroperitoneum. Surgery is currently the only treatment that can lead to a cure. However, the optimal surgical strategy for tumors in the cardiac area of the stomach, especially tumors invading the lower esophagus, remains controversial<sup>[6]</sup>. The development of effective therapeutic strategies for these tumors requires information on patient characteristics, patterns of lymph node metastasis, and the efficacy of lymph node dissection. Adenocarcinoma of the cardia generally has a low curative resection rate and a poor prognosis; worse than carcinoma of the other regions of the stomach, mainly because the disease is at a more advanced stage at diagnosis<sup>[6-8]</sup>. The 5-year survival rate in resected cases is  $\leq 20\%$ <sup>[9]</sup>.

The role of lymphadenectomy in GC surgery has been hotly debated during the past three decades. Although there is still no standard approach, it is obvious that an adequate lymphadenectomy, removing all the possible metastatic nodes, remains a milestone in GC surgery<sup>[10]</sup>. The most recent edition of the tumor, node, metastasis (TNM) classification states that at least 15 lymph nodes must be examined to form an accurate eval-

uation of the node status. The optimal extent of lymphadenectomy (D2) for this cancer has been defined in the Japanese Classification of Gastric Carcinoma<sup>[11]</sup>, based on the retrospective historical data of the involved nodes in patients with gastric carcinoma. The optimal extent of lymph node dissection for Siewert type II esophagogastric junction (EGJ) carcinoma is poorly defined in this classification. Rüdiger Siewert *et al*<sup>[12]</sup> uncovered the distribution of metastatic nodes in patients with type II adenocarcinoma. In their cohort of 186 patients, they found that the disease mainly involved the paracardial and lesser curve nodes, followed in frequency by the nodes in the lower mediastinum, and suprapancreatic nodes and nodes along the greater curve were involved in patients with Siewert type II EGJ cancers. Furthermore, they found positive parapyloric nodes in three of their patients, which lends support to their recommended strategy of extended total gastrectomy for type II EGJ carcinoma.

Therefore, in the present study, we reevaluated retrospectively the clinicopathological features and distribution of metastatic nodes in a two-center cohort of 146 patients with gastric cardiac adenocarcinoma. Univariate and multivariate analyses were applied to confirm the clinicopathological factors associated with lymph node metastases, and to provide a basis for choosing the optimal surgical treatment and for determining the appropriate range of lymph node dissection.

## MATERIALS AND METHODS

### Patients

Data were collected from a prospectively maintained database of patients with histologically confirmed gastric cardiac carcinoma who had curative gastrectomy (R0) with lymphadenectomy in the Department of Surgery, Xin Hua Hospital and Rui Jin Hospital of Shanghai Jiaotong University Medical School between November 2001 and May 2012. The clinicopathological characteristics and lymph node metastasis of gastric cardiac adenocarcinoma were compared in male and female patients (Table 1).

### Surgery

All operations were performed with curative intent. Curative surgery was defined as the removal of all gross tumor and the demonstration of tumor-negative surgical margins by microscopic examination of the entire circumference. Subtotal or total gastrectomy was performed according to the tumor size, tumor location, and the status of the resection margins. Proximal gastrectomy involved resection of the proximal half of the stomach *via* an abdominal or thoracic approach, with an esophago-gastric anastomosis. Following total gastrectomy with D2 lymph node dissection, an esophagojejunostomy was used routinely for Roux-en-Y reconstruction. Proximal resection margins were evaluated intraoperatively to confirm freedom from disease. Resection of adjacent organs was undertaken to achieve clear margins when deemed necessary. Both the surgical procedure and the extent of

**Table 1 Demographics and clinicopathological features of gastric cardiac adenocarcinoma**

Factors	Total ( <i>n</i> = 146)	Sex		<i>P</i> value
		Female ( <i>n</i> = 35)	Male ( <i>n</i> = 111)	
Age (yr)				0.668
< 60	46	10	36	
≥ 60	100	25	75	
Type of gastrectomy				0.776
Total	53	12	41	
Proximal gastrectomy	93	23	70	
Splenectomy				0.102
Presence	8	0	8	
Absence	138	35	103	
Tumor size (cm)				0.717
< 2	2	0	2	
2-5	93	23	70	
> 5	51	12	39	
Gross appearance				0.931
Type 0	6	2	4	
Type 1	13	3	10	
Type 2	105	26	79	
Type 3	13	2	11	
Type 4	9	2	7	
Tumor differentiation				0.389
Differentiated	76	16	60	
Undifferentiated	70	19	51	
Lymph nodes retrieved	22.88 ± 9.162	23.06 ± 9.449	22.78 ± 9.089	0.602
Pathological tumor depth				0.729
T1	8	2	6	
T2	16	2	14	
T3	4	1	3	
T4	118	30	88	
Node status (TNM)				0.665
pN <sub>0</sub>	43	11	32	
pN <sub>1</sub>	27	4	23	
pN <sub>2</sub>	29	8	21	
pN <sub>3</sub>	47	12	35	
pTNM staging				0.445
I	15	2	13	
II	37	11	26	
III	94	22	72	
IV				
Lymphatic invasion				0.694
Positive	24	5	19	
Negative	122	30	92	
Venous invasion				0.393
Positive	5	2	3	
Negative	141	33	108	
Nerve invasion				0.350
Positive	22	7	15	
Negative	124	28	96	
Esophageal involvement				0.497
Presence	31	6	25	
Absence	115	29	86	

TNM: Tumour, node, metastasis.

lymph node dissection were based on the recommendations of the Japanese GC treatment guidelines<sup>[13]</sup>. No patient received neoadjuvant chemotherapy or postoperative radiotherapy.

### Pathological examination

In both hospitals, the surgical team immediately examined the lymph nodes macroscopically, which were then divid-

ed and classified into lymph node stations, as defined by the Japanese Classification of Gastric Carcinoma<sup>[14]</sup>. No size limitation was imposed for lymph node harvesting. Specimens were fixed in formalin, stained with hematoxylin and eosin, and sent for histopathological evaluation, following which the number of histologically confirmed lymph nodes was recorded for each lymph node station. Each lymph node was embedded in paraffin and at least two sections were taken. Immunohistochemistry for micrometastasis was not performed.

Tumor size was recorded as the maximum diameter. The depth of infiltration was measured at the deepest point of penetration of the cancer cells. In this study, we referred to the classifications established by the Japanese Classification of Gastric Carcinoma: 3<sup>rd</sup> English edition<sup>[14]</sup>, which define T1 as a tumor confined to the mucosa (M) or submucosa (SM); T2 as a tumor that invades the muscularis propria (MP); T3 as a tumor that invades the subserosa (SS); and T4 as tumor invasion that is contiguous to or exposed beyond the serosa (SE) or tumor invades adjacent structures (SI). The macroscopic type was classified as type 0 (superficial), type 1 (mass), type 2 (ulcerative), type 3 (infiltrative ulcerative), type 4 (diffuse infiltrative) or type 5 (unclassifiable). We evaluated the tumor histology according to the classification established by the Japanese Research Society for GC<sup>[11]</sup>. Well- and moderately differentiated tubular adenocarcinoma, and papillary adenocarcinoma were classified as differentiated-type carcinomas; and poorly differentiated adenocarcinoma, signet ring cell carcinoma and mucinous carcinoma were classified as undifferentiated-type carcinomas.

The nodal classification was classified into four groups: pN<sub>0</sub>, no metastasis; pN<sub>1</sub>, one or two positive regional lymph nodes; pN<sub>2</sub>, 3-6 positive regional lymph nodes; and pN<sub>3</sub>, ≥ 7 positive regional lymph nodes. We conducted the tumor staging according to the Japanese Classification of Gastric Carcinoma: 3<sup>rd</sup> English edition<sup>[14]</sup>. The ratio of lymph node metastasis was calculated by determining the number of patients with a metastasized lymph node in a particular station divided by the number of patients who underwent dissection of that lymph node. The metastatic incidence is the ratio of metastatic nodes to the total number of dissected nodes and was recorded for each nodal station for all regional lymph nodes.

### Ethics

This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The Institutional Review Board of Shanghai Jiaotong University gave ethical approval for this study. All patients provided written informed consent.

### Statistical analysis

Descriptive data are presented as the mean ± SD. For between group comparisons, continuous variables were analyzed using Student's *t* test, and categorical variables with the  $\chi^2$  test. Factors found to be significant (*P* <

**Table 2** Univariable analysis of lymph nodes metastasis in gastric cardiac cancer and clinicopathological factors

Factors	Total (n = 146)			Female (n = 35)			Male (n = 111)		
	LN+	LN-	P value	LN+	LN-	P value	LN+	LN-	P value
Age (yr)			0.927			0.134			0.353
< 60	33	13		5	5		28	8	
≥ 60	71	29		19	6		52	23	
Tumor size (cm)			0.011			0.554			0.011
< 2	0	2		0	0		0	2	
2-5	62	31		15	8		47	23	
> 5	42	9		9	3		33	6	
Gross appearance			0.000			0.211			0.000
Type 0	0	6		0	2		0	4	
Type 1	5	8		2	1		3	7	
Type 2	82	23		19	7		63	16	
Type 3	10	3		2	0		8	3	
Type 4	7	2		1	1		6	1	
Tumor differentiation			0.000			0.150			0.000
Differentiated	44	32		9	7		35	25	
Undifferentiated	60	10		15	4		45	6	
Pathological tumor depth			0.000			0.051			0.000
T1	1	7		0	2		1	5	
T2	8	8		2	0		6	8	
T3	3	1		0	1		3	0	
T4	92	26		22	8		70	18	
Lymphatic invasion			0.001			0.102			0.003
Positive	24	0		5	0		19	0	
Negative	80	42		19	11		61	31	
Venous invasion			0.659			0.324			0.832
Positive	4	1		2	0		2	1	
Negative	100	41		22	11		78	30	
Nerve invasion			0.966			0.856			0.972
Positive	15	6		5	2		11	4	
Negative	88	36		19	9		69	27	
Esophageal involvement			0.192			0.912			0.131
Presence	25	6		4	2		21	4	
Absence	79	36		20	9		59	27	

0.05) in univariate analysis were included in subsequent multivariate logistic regression analysis, to identify independent variables associated with lymph node metastases. All statistical analyses were undertaken using SPSS for Windows, version 18.0 (SPSS, Chicago, IL, United States). For all analyses,  $P < 0.05$  was considered statistically significant.

## RESULTS

### *Demographics and clinicopathological features of gastric cardiac adenocarcinoma*

The clinicopathological characteristics of gastric cardiac cancer are illustrated in Table 1. Among the 146 patients, there were 111 men and 35 women, ranging in age from 16 to 84 years (mean  $63.9 \pm 11.6$  years). Surgical procedures comprised 93 proximal gastrectomies and 53 total gastrectomies. Splenectomy was required in 8 (5.5%) of the 146 patients undergoing curative resections. The total splenectomy patients were all male. Mean tumor length was 5.54 cm. Of the 146 patients, 6 (4.1%), 13 (8.9%), 105 (71.9%), 13 (8.9%) and 8 (5.8%) were type 0, 1, 2, 3 and 4, respectively. Tumors were differentiated in 76 patients and undifferentiated in 70. The number of lymph

nodes retrieved was  $22.88 \pm 9.16$ , and 104 patients had positive lymph node metastases (71.2%). There were eight cases with a T1 tumor, 16 with a T2 tumor, 4 with a T3 tumor, and 118 with a T4 tumor. Lymph node involvement according to the Japanese Classification of Gastric Carcinoma: 3<sup>rd</sup> English edition<sup>[14]</sup> included 43 patients with N0 disease, 27 with N1 disease, and 76 with N2-3 disease (Table 2). Evidence of lymphatic invasion, venous invasion and neural invasion was seen in 24 (16.4%), 5 (3.4%) and 22 patients (15.1%), respectively. On pathological examination, the tumors of 31 patients (21.2%) were found to have invaded the lower esophagus. None of the clinicopathological factors, such as age, type of gastrectomy, tumor size, gross appearance, tumor differentiation, pathological tumor depth, node status, pTNM staging, lymphatic invasion, venous invasion, nerve invasion and esophagus involvement were different between male and female patients ( $P > 0.05$ ).

### *Univariate analysis of lymph node metastasis in gastric cardiac cancer and clinicopathological factors*

Univariate analysis was performed on the relationship between lymph node metastases and clinicopathological factors. The findings revealed a close relationship between

**Table 3** Univariate analysis of lymph node metastases in gastric cardiac adenocarcinoma and clinicopathological factors for sex difference

Factors	Female LN+ (n = 24)	Male LN+ (n = 80)	P value
Age (yr)			0.191
< 60	15.20%	84.80%	
≥ 60	26.80%	73.20%	
Tumor size (cm)			0.743
< 2	0.00%	0.00%	
2-5	24.20%	75.80%	
> 5	21.40%	78.60%	
Gross appearance			0.961
Type 0	0.00%	0.00%	
Type 1	40.00%	60.00%	
Type 2	23.20%	76.80%	
Type 3	20.00%	80.00%	
Type 4	14.30%	85.70%	
Tumor differentiation			0.587
Differentiated	20.50%	79.50%	
Undifferentiated	25.00%	75.00%	
Pathological tumor depth			0.627
T1	0.00%	100.00%	
T2	25.00%	75.00%	
T3	0.00%	100.00%	
T4	23.90%	76.10%	
Lymphatic invasion			0.766
Positive	20.80%	79.20%	
Negative	23.80%	76.20%	
Venous invasion			0.192
Positive	50.00%	50.00%	
Negative	22.00%	78.00%	
Nerve invasion			0.399
Positive	31.30%	68.70%	
Negative	21.60%	78.40%	
Esophageal involvement			0.335
Presence	16.00%	84.00%	
Absence	25.30%	74.70%	

tumor size, gross appearance, differentiation, pathological depth, lymphatic invasion and lymph node metastases in all patients ( $P = 0.011$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$  and  $P = 0.001$ , respectively) and in male patients ( $P = 0.011$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$  and  $P = 0.003$ , respectively). However, there was no obvious correlation between lymph node metastases and clinicopathological features in female patients, nor between male and female patients (Table 3).

#### Multivariate analysis of lymph node metastases in gastric cardiac cancer for the entire study population and male patients

Multivariate analysis revealed that only tumor differentiation was an independent risk factor for lymph node metastases in gastric cardiac cancer for the entire study population ( $P = 0.001$ ). Tumor size, gross appearance, pathological depth and lymphatic invasion had no significant effect on nodal involvement rates (Table 4). Multivariate analysis revealed that tumor differentiation and pathological depth were independent risk factors for lymph node metastases in gastric cardiac cancer for male patients ( $P = 0.001$ ,  $P = 0.020$ ). Tumor size, gross appearance and lymphatic invasion had no significant effect

on nodal involvement rates (Table 4).

#### Relationship between sex and number of metastatic lymph nodes

There was no significant difference between female and male patients in terms of the number of retrieved lymph nodes, using the independent sample  $t$  test ( $P = 0.878$ ). The number of metastatic lymph nodes in female patients was higher than that in male patients ( $6.20 \pm 7.49$  vs  $4.84 \pm 5.44$ ). However, the difference was not significant ( $P = 0.243$ ).

**Retrieved lymph nodes, lymph node metastases, lymph node metastasis ratios and incidence for involved lymph nodes at each station in gastric cardiac adenocarcinoma**  
Lymph nodes ( $n = 3340$ , median 22.88; range 15-62) were removed from the 146 patients and examined, and 754 (median 5.16; range 0-30) were metastatic. For female patients, 807 (median 23.06; range 15-61) lymph nodes were examined and 217 (median 6.20; range 0-30) contained metastases. For male patients, 2533 (median 22.82; range 15-62) lymph nodes were examined and 537 (median 4.84; range 0-26) contained metastases (Table 5).

According to the Japanese Classification of Gastric Carcinoma: 3<sup>rd</sup> English edition<sup>[14]</sup>, 103 cases (70.5%) were at N1, 23 cases (15.8%) at N2, 15 cases (10.3%) at N3, and four cases (2.7%) at M. A direct skip to N3, without moving through N2, occurred in 10 cases (6.8%). There were no skips to N2 without going through N1. Nodal metastases were frequent in the abdominal nodes, followed in frequency by involvement of the No. 3 (59.6%), No. 1 (26.7%), No. 2 (18.5%), and No. 4 (16.4%) nodes, and thereafter by mediastinal lymph nodes, which were affected only in a small number in our series (No. 110, 0.7%). The frequency of the metastatic involvement of the supra- and infra-pyloric nodes was low (4.1% and 3.4%, respectively), and no cases with metastasis to Nos. 13-15 were found. Only four patients received station No. 16 lymph node dissection, and three of them had metastasis (Table 5).

The extent of metastases in female cases was as follows: 24 cases were at N1 (16.4%, 24/146), representing a metastatic rate of 68.6% (24/35); 5 cases were at N2 (3.4%, 5/146), with a metastatic rate of 14.3% (5/146); and 4 cases were found at N3 (2.7%, 4/146), with a metastatic rate of 11.4% (4/146). The extent of metastases in male patients was as follows: 79 cases (54.1%) occurred at N1, with a metastatic rate of 71.2%; 18 cases occurred at N2 (12.3%), with an incidence of 16.2%; and 11 cases occurred at N3 (7.5%), with an incidence of 9.9% (Table 5).

**Correlation between lymph node metastases at pN<sub>1</sub>, pN<sub>2</sub> and pN<sub>3</sub> and clinicopathological factors, using the Japanese GC association classification for the entire study population and between male and female patients**  
Univariate analysis of variance revealed a close relationship between tumor size, gross appearance, differentiation, pathological depth and lymphatic invasion and

**Table 4 Multivariate analysis of lymph node metastases in gastric cardiac cancer for the entire study population**

Multivariate analysis	B	SE	$\chi^2$ value	P value	OR	95%CI	
						Lower	Upper
Entire study population							
Tumor size	0.010	0.528	0.000	0.985	0.010	0.359	2.843
Gross appearance	-0.169	1.166	0.021	0.885	0.845	0.086	8.302
Tumor differentiation	1.806	0.522	11.981	0.001	6.084	2.188	16.912
Tumor depth	0.464	0.299	2.400	0.121	1.590	0.884	2.858
Lymphatic invasion	20.207	7720.675	0.000	0.998	5.967E8	0.000	
Constant	-3.181	1.664	3.656	0.056	0.042		
Male patients							
Tumor size	0.594	0.707	0.705	0.401	1.810	0.453	7.233
Gross appearance	-1.420	1.442	0.969	0.325	0.242	0.014	4.085
Tumor differentiation	2.525	0.749	11.375	0.001	12.493	2.880	54.199
Tumor depth	0.838	0.359	5.448	0.020	2.313	1.144	4.676
Lymphatic invasion	20.295	8351.751	0.000	0.998	6.514E8	0.000	
Constant	-5.010	2.212	5.132	0.023	0.007		

**Table 5 Number of retrieved lymph nodes, lymph node metastases, lymph node metastasis ratios, and incidence at each station**

Node station	pN category	Number of dissected nodes			Number of metastasis nodes			Incidence of lymph node metastasis			Ratio of lymph node metastasis		
		T	F	M	T	F	M	T	F	M	T	F	M
No. 1	pN <sub>1</sub>	448	111	337	100	31	69	22.30%	26.90%	20.50%	26.70%	31.40%	24.30%
No. 2	pN <sub>1</sub>	249	67	182	54	12	42	21.70%	17.90%	23.10%	18.50%	20.00%	18.00%
No. 3	pN <sub>1</sub>	1308	334	974	404	100	304	30.90%	29.90%	31.20%	59.60%	60.00%	59.50%
No. 4	pN <sub>1</sub>	589	136	453	87	45	42	14.80%	33.10%	9.30%	16.40%	22.90%	14.40%
No. 5	pN <sub>3</sub>	39	9	30	11	7	4	28.20%	77.80%	13.30%	4.10%	5.70%	3.60%
No. 6	pN <sub>3</sub>	146	33	113	13	9	4	8.90%	27.30%	3.50%	3.40%	8.60%	1.80%
No. 7	pN <sub>2</sub>	176	49	127	32	8	24	18.20%	16.30%	18.90%	11.60%	8.60%	12.60%
No. 8	pN <sub>2</sub>	108	26	82	13	3	10	12.00%	11.50%	12.20%	4.10%	5.70%	3.60%
No. 9	pN <sub>2</sub>	39	8	31	15	2	13	38.50%	25.00%	41.90%	4.10%	2.90%	4.50%
No. 10	pN <sub>2</sub>	37	11	26	8	0	8	21.60%	0.00%	30.80%	1.40%	0.00%	1.80%
No. 11	pN <sub>2</sub>	36	6	30	5	0	5	13.90%	0.00%	16.70%	2.70%	0.00%	3.60%
No. 12	pN <sub>2</sub>	45	6	39	5	0	5	11.10%	0.00%	12.80%	1.40%	0.00%	1.80%
No. 13	M	8	0	8	0	0	0	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
No. 14	M	30	2	28	0	0	0	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
No. 15	M	13	5	8	0	0	0	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
No. 16	M	32	0	32	6	0	6	18.80%	0.00%	18.80%	2.10%	0.00%	2.70%
No. 110	M	37	4	33	1	0	1	2.70%	0.00%	3.00%	0.70%	0.00%	0.90%
Total		3340	807	2533	754	217	537	22.70%	26.90%	21.30%			

lymph node metastases at pN<sub>1</sub> in all patients ( $P = 0.020$ ,  $P = 0.000$ ,  $P = 0.000$ ,  $P = 0.000$  and  $P = 0.001$ , respectively) and male patients ( $P = 0.021$ ,  $P = 0.000$ ,  $P = 0.001$ ,  $P = 0.001$  and  $P = 0.002$ , respectively) (Table 6). However, there was no obvious correlation between lymph node metastases and clinicopathological features in female patients (Table 6) and between male and female patients (Table 7).

There was obvious relationship between the lymphatic invasion and lymph node metastases at pN<sub>2</sub> in all patients ( $P = 0.048$ ) and male patients ( $P = 0.046$ ) (Table 6). There was no significant correlation between clinicopathological features and the presence of lymph node metastases at pN<sub>2</sub> in female patients (Table 6) nor between male and female patients (Table 7).

There was an obvious relationship between lymphatic invasion and nerve invasion and lymph node metastases at pN<sub>3</sub> in all patients ( $P = 0.009$ ,  $P = 0.001$ ) (Table 6). There was a significant correlation between lymphatic invasion in female patients (Table 6) and neural invasion in

male patients (Table 6) and the presence of lymph node metastases at pN<sub>3</sub>. There was no significant correlation between clinicopathological features and the presence of lymph node metastases at pN<sub>3</sub> between male and female patients (Table 7).

## DISCUSSION

According to some clinicians, true carcinoma of the cardia may be considered a distinct clinical entity, with different biological behavior and a more aggressive natural history than subcardial gastric carcinoma<sup>[15-17]</sup>. Strangely enough, the location, extent and even the existence of the gastric cardia are controversial<sup>[18]</sup>. Anatomists have applied the term cardia to that part of the stomach that lies around the orifice of the tubular esophagus. The American Joint Committee on Cancer describes the EGJ as the first part of the stomach, which is located immediately below the diaphragm and is often called the cardia<sup>[19]</sup>. The definition of the cardia commonly employed in Japan is

Table 6 Correlation between lymph node metastases at pN1, pN2 and pN3 and clinicopathological factors

Characteristics	pN1				pN2				pN3																			
	Total		Female		Male		Total		Female		Male																	
	LN+	LN-	P value	LN+	LN-	P value	LN+	LN-	P value	LN+	LN-	P value																
Age (yr)																												
< 60	33	13	0.830	5	5	0.134	28	8	0.287	11	35	0.066	2	8	0.541	9	27	0.082	4	42	0.670	0	10	0.179	4	32	0.769	
≥ 60	70	30		19	6		51	24		12	88		3	22		9	66		11	89		4	21		7	68		
Tumor size (cm)																												
< 2.0	0	2	0.020	0	0	0.554	0	2	0.021	0	2	0.151	0	0	0.191	0	2	0.314	0	2	0.095	0	0	0.068	0	2	0.345	
2.0-5.0	62	31		15	8		47	23		11	82		2	21		9	61		6	87		1	22		5	65		
> 5.0	41	10		9	3		32	7		12	39		3	9		9	30		9	42		3	9		6	33		
Gross appearance																												
Type 0	0	6	0.000	0	2	0.211	0	4	0.000	0	6	0.674	0	2	0.513	0	4	0.748	0	6	0.395	0	2	0.428	0	4	0.560	
Type 1	5	8		2	1		3	7		1	12		0	3		1	9		0	13		0	3		0	10		
Type 2	81	24		19	7		62	17		19	86		4	22		15	64		13	92		3	23		10	69		
Type 3	10	3		2	0		8	3		2	11		1	1		1	10		2	11		1	1		1	10		
Type 4	7	2		1	1		6	1		1	8		0	2		1	6		0	9		0	2		0	7		
Tumor differentiation																												
Differentiated	44	32	0.000	9	7	0.150	35	25	0.001	8	68	0.071	2	14	0.782	6	54	0.054	6	70	0.324	2	14	0.855	4	56	0.215	
Undifferentiated	59	11		15	4		44	7		15	55		3	16		12	39		9	61		2	17		7	44		
Pathological tumor depth																												
T1	1	7	0.000	0	2	0.051	1	5	0.001	0	8	0.360	0	2	0.808	0	6	0.406	0	8	0.265	0	2	0.861	0	6	0.363	
T2	8	8		2	0		6	8		1	15		0	2		1	13		0	16		0	2		0	14		
T3	3	1		0	1		3	0		1	3		0	1		1	2		0	4		0	1		0	3		
T4	91	27		22	8		69	19		21	97		5	25		16	72		15	103		4	26		11	77		
Lymphatic invasion																												
Yes	24	0	0.001	5	0	0.102	19	0	0.002	7	17	0.048	1	4	0.693	6	13	0.046	6	18	0.009	2	3	0.030	4	15	0.074	
No	79	43		19	11		60	32		16	106		4	26		12	80		9	113		2	28		7	85		
Venous invasion																												
Yes	4	1	0.637	2	0	0.324	2	1	0.861	1	4	0.791	1	1	1.000	0	3	0.440	1	4	0.466	0	2	0.601	1	2	0.169	
No	99	42		22	11		77	31		22	119		4	29		18	90		14	127		4	29		10	98		
Nerve invasion																												
Yes	15	7	0.792	5	2	0.856	10	5	0.679	4	18	0.734	1	6	1.000	3	12	0.669	7	15	0.001	2	5	0.111	5	10	0.001	
No	88	36		19	9		69	27		19	105		4	24		15	81		8	116		2	26		6	90		
Esophageal involvement																												
Yes	25	6	0.165	4	2	0.912	21	4	0.108	2	29	0.109	0	6	0.272	2	23	0.205	4	27	0.587	1	5	0.658	3	22	0.691	
No	78	37		20	9		58	28		21	94		5	24		16	70		11	104		3	26		8	78		

the area within 2 cm above and below the EGJ, and tumors whose center is situated in this area are considered to be cancer of the cardia; such cancers are distinguished from upper GCs. Siewert *et al*<sup>[20]</sup> proposed a topographical classification for cardiac carcinomas. In contrast to the previously described classification system, Siewert and Stein<sup>[20]</sup> attempted to resolve the problem of splitting up EGJ tumors into esophageal and gastric tumors by creating a third entity. These tumours show a high rate of early lymphatic dissemination and lymph node metastases<sup>[12,21]</sup> and are usually related to poorer prognosis<sup>[12,21]</sup>. The reasons for this sudden increase of gastric cardia carcinomas are not clear, but changing risk factors such as smoking, alcohol use, presentation at a more advanced stage, salty foods, pollution and increases in gastroesophageal reflux diseases might explain it partially<sup>[5,7,23]</sup>.

**Table 7 Correlation between lymph node metastases at pN1, pN2 and pN3 and clinicopathological factors for sex difference**

Clinicopathological factors	pN1			pN2			pN3		
	Female LN+	Male LN+	P value	Female LN+	Male LN+	P value	Female LN+	Male LN+	P value
Age (yr)			0.179			0.692			0.159
< 60	5 (15.2)	28 (84.8)		2 (18.2)	9 (81.8)		0 (0.0)	4 (100.0)	
≥ 60	19 (27.1)	51 (72.9)		3 (25.0)	9 (75.0)		4 (36.4)	7 (63.6)	
Tumor size (cm)			0.792			0.692			0.475
< 2.0	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
2.0-5.0	15 (24.2)	47 (75.8)		2 (18.2)	9 (81.8)		1 (16.7)	5 (83.3)	
> 5.0	9 (22.0)	32 (78.0)		3 (25.0)	9 (75.0)		3 (33.3)	6 (66.7)	
Gross appearance			0.960			0.472			0.423
Type 0	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Type 1	2 (40.0)	3 (60.0)		0 (0.0)	1 (100.0)		0 (0.0)	0 (0.0)	
Type 2	19 (23.5)	62 (76.5)		4 (21.1)	15 (78.9)		3 (23.1)	10 (76.9)	
Type 3	2 (20.0)	8 (80.0)		1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)	
Type 4	1 (14.3)	6 (85.7)		0 (0.0)	1 (100.0)		0 (0.0)	0 (0.0)	
Tumor differentiate			0.555			0.782			0.634
Differentiated	9 (20.5)	35 (79.5)		2 (25.0)	6 (75.0)		2 (33.3)	4 (66.7)	
Undifferentiate	15 (25.4)	44 (74.6)		3 (20.0)	12 (80.0)		2 (22.2)	7 (77.8)	
Pathological tumor depth			0.624			0.738			NS
T1	0 (0.0)	1 (100.0)		0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
T2	2 (25.0)	6 (75.0)		0 (0.0)	1 (100.0)		0 (0.0)	0 (0.0)	
T3	0 (0.0)	3 (100.0)		0 (0.0)	1 (100.0)		0 (0.0)	0 (0.0)	
T4	22 (24.2)	69 (75.8)		5 (23.8)	16 (76.2)		4 (26.7)	11 (73.3)	
Lymphatic invasion			0.744			0.567			0.634
Positive	5 (20.8)	19 (79.2)		1 (14.3)	6 (85.7)		2 (33.3)	4 (66.7)	
Negative	19 (24.1)	60 (75.9)		4 (25.0)	12 (75.0)		2 (22.2)	7 (77.8)	
Venous invasion			0.198			0.052			0.533
Positive	2 (50.0)	2 (50.0)		1 (100.0)	0 (0.0)		0 (0.0)	1 (100.0)	
Negative	22 (22.2)	77 (77.8)		4 (18.2)	18 (81.8)		4 (28.6)	10 (71.4)	
Nerve invasion			0.247			0.862			0.733
Positive	5 (35.7)	9 (64.3)		1 (25.0)	3 (75.0)		2 (33.3)	4 (66.7)	
Negative	19 (21.6)	69 (78.4)		4 (21.1)	15 (78.9)		2 (25.0)	6 (75.0)	
Esophageal involvement			0.321			0.435			0.930
Presence	4 (16.0)	21 (84.0)		0 (0.0)	2 (100.0)		1 (25.0)	3 (75.0)	
Absence	20 (25.6)	58 (74.4)		5 (23.8)	16 (76.2)		3 (27.3)	8 (72.7)	

It is crucial that the therapeutic strategy for gastric cardiac adenocarcinoma be clarified through evaluation of both the pattern of lymph node metastasis and the efficacy of lymph node dissection in this region. According to Siewert *et al.*<sup>[20]</sup>, metastases already exist in 72.0% of the cases at the time of surgery on tumors of the distal esophagus and the cardia. Lymphogenous metastases were present in 73.5% of the cases in our study. The cause of the frequent invasion of lymph nodes is the density of the lymph duct supply both to the stomach and to the lower esophagus, such that the cancers at the EGJ invade the regional lymph nodes concerned at an early stage<sup>[24]</sup>.

Previous studies have proved that the number of lymph nodes retrieved has a significantly impact in pN category, which resulted in a “stage-migration” phenomenon<sup>[25-27]</sup>; therefore, in the present study the quality of lymphadenectomy was adequate because the median number of resected lymph nodes was clearly more than 15, as recommended by the Japanese Classification of Gastric Carcinoma: 3<sup>rd</sup> English edition<sup>[14]</sup>. Furthermore, the median number of 23 lymph nodes in our study is comparable with other prospective studies on the treatment of GC<sup>[27,28]</sup>. The number of dissected lymph nodes is closely associated with the pathological stages and prognosis. A

population-based study by Bouvier *et al.*<sup>[29]</sup> showed that the error rate was 47.1% if the pathological stages were classified according to the identical TNM stages for the patients with < 10 or > 15 detected lymph nodes. Thus, the pathological stages are not reliable for patients with < 10 detected lymph nodes in GC surgery. On TNM stages, Union for International Cancer Control version 5 states that the number of dissected lymph nodes in advanced GC must be ≥ 15 to ensure the reliability of pathological stages and prognosis judgment. In a study reported by Karpeh *et al.*<sup>[30]</sup>, 27 patients with GC classified as stage II and III disease, and having < 15 lymph nodes examined, had significantly lower 5-year survival rates than those who had ≥ 15 lymph nodes examined. In a similar analysis, Bouvier *et al.*<sup>[29]</sup> concluded that > 10 lymph nodes should be analyzed per specimen to allow for valid N staging.

The sex distribution in this study showed an absolute male predominance (3.2:1) in gastric cardiac adenocarcinoma, which is similar to previous studies<sup>[23,31,32]</sup>. The sex ratio for cancer of the pylorus is only 1.5<sup>[33]</sup>. Although the exact reason for the male predominance of this type of cancer remains unknown, it seems to be a definite feature of this type of tumor, irrespective of the origin of the population<sup>[7,34]</sup>. Some scholars pointed out that this

may be because sex hormones such as estrogen affect the incidence rate of GC<sup>[35,36]</sup>. In our group, 98.6% of patients had tumors > 2 cm. Larger tumors have higher rates of lymph node metastases. Of the 104 cases with lymph node metastases, all the tumor sizes were > 2 cm, accounting for all metastases. Morphological classification was mainly of the ulcerative type (71.9%). Otherwise, type 0, 1, 3 and 4 accounted for 4.1%, 8.9%, 8.9% and 5.5%, respectively. Histologically, there were slightly more undifferentiated tumors (52.1%) than differentiated tumors (47.9%), and > 83.6% of patients had T3 or T4 tumors.

Toward the latter, the seventh edition of the TNM classification of malignant tumors defines rules for classifying carcinomas arising within the vicinity of the EGJ to end the imprecise regulation of earlier editions, where carcinomas around the EGJ could be staged according to either the classification of esophageal carcinomas or the classification of gastric carcinomas. However, neither of the two staging systems has proven to be clearly superior to the other, and neither of them is perfect for so-called cardiac adenocarcinomas. For the N classification of the so-called cardiac adenocarcinomas, both schemes are monotone and distinct, with continuously decreasing and significantly different prognosis with an increasing number of lymph node metastases<sup>[37]</sup>. Huang<sup>[38]</sup> pointed out that the Version 7 manual would predict the prognosis of patients more effectively than the Version 6 manual according to the staging of GC. The staging of lymph nodes (pN) can predict the prognosis better than the invasion depth of cancer tissue (pT), while the lymph node status in the axial area of the celiac artery is particularly critical. The Version 7 manual defined the EGJ-involved gastric cardia cancer staging improperly and this should be corrected. Of course, their research results are to be updated and verified with more large-sample studies. Huang *et al.*<sup>[39]</sup> postulated that type II EGJ adenocarcinomas are more adequately staged as GC by the seventh edition of the American Joint Committee on Cancer classification.

Many researchers have attempted to investigate the relationship between nodal involvement and clinicopathological factors. The factors related to lymph node metastasis include age, sex, clinical staging of tumor, pathological tissue type, invasion depth of lesion, tumor size, and typing. As expected, we found tumor characteristics such as tumor size, gross appearance, differentiation, pathological depth and lymphatic invasion were associated with lymph node metastases in all patients and male patients, and could represent a selection indicator of lymph node dissection. However, there was no obvious correlation between lymph node metastases and clinicopathological features in female patients. In gastric cardiac adenocarcinoma, the clinicopathological features and lymph node metastasis patterns did not differ significantly between male and female patients. These results were similar to those reported by previous studies<sup>[40]</sup>. Male patients had lymph node metastasis in 72.1%;

slightly higher than that in female patients. The present study discovered that the metastasis rate of lymph nodes increased with the maximum diameter of the lesion; nevertheless, it is not advisable to simply take the tumor size as the correlation factor for predicting the lymph node metastasis because of variations in the period of tumor growth. Borrmann typing is also related to lymph node metastasis. The metastasis rate of lymph nodes in type III and IV GC was significantly higher than in type I and II in this paper. This could be explained by the main invasion growth of the former types and the limited growth of the later types, because weak or strong invasion ability may lead to differences in the metastasis rate of lymph nodes. Histological type is closely related to nodal status. In our group, the rate of lymph node metastases in undifferentiated tumors was higher than that observed in differentiated cancer: 85.7% (60/70) and 67.9% (44/76), respectively. The tumor differentiation extent decides the biological behavior of GC. A larger extent of cell differentiation possibly causes a larger metastasis rate of lymph nodes. Some scholars have found that poorly differentiated GC cells produced more type IV collagenase, which can degrade the basilar membrane, reduce the ability to resist cancer cell infiltration, and cause the rate of lymph node metastasis to be higher than that for differentiated adenocarcinoma. Moreover, there is an increasing rate of node involvement as the T stage increases; in our series, 12.5% of T1, 50.0% of T2, 75.0% of T3 and 78.0% of T4 cases had positive nodes. This suggests a correlation between T stage factor and the presence of positive nodes. The results of this study showed that lymphatic duct invasion is closely related to the lymph node metastasis; the metastasis rate of lymph nodes was up to 100% in the LVI (+) group, but 0% in the LVI (-) groups. Many studies have shown that metastasis of lymphatic duct invasion occurs before lymph node metastasis. The presence of lymphatic duct invasion or cancer cells indicates the prophase of lymph node metastasis or a manifestation of lymph node metastasis. The above factors should be the focus of preoperative gastric cardia treatment options. The appropriate degree of lymph node dissection must be selected to improve the surgical efficacy in gastric cardia cancer.

In this study, multivariate analysis revealed that tumor differentiation was the only independent risk factor for lymph node metastases in all patients, and revealed that tumor differentiation and pathological depth were independent risk factors for lymph node metastases in male patients. By logistic methods, Liu *et al.*<sup>[41]</sup> also confirmed that the tumor length, invasion depth, blood vessel invasion and specimen stump had a significant effect on lymph node metastasis. With the increase of tumor length and invasion depth, the appearance of blood vessel invasion and specimen stump cancer cells, the risk of lymph node metastasis increased significantly.

The new nodal staging in the 7<sup>th</sup> TNM classification is based on the number of metastatic nodes. In our group, all 146 cases of gastric cardiac adenocarcinoma

received radical gastrectomy. Postoperatively, 3340 regional lymph nodes were located. Seven hundred and fifty-four lymph nodes were found in 104 cases with lymph node metastases - an average of 7.25 per case. It had been considered that all the regional nodes of the stomach were potentially involved in metastasis in patients with adenocarcinoma of the gastric cardia<sup>[42]</sup>. Lymphogenous metastasis by cancer of the cardia frequently affects the lymph nodes at the greater and lesser curvature of the stomach. Less frequent involvement of the lymph nodes at right cardiac and left cardiac lymph nodes has been observed<sup>[39,43,44]</sup>. In line with previous findings<sup>[12,45,46]</sup>, the Mine *et al*<sup>[47]</sup> confirmed that nodal station numbers 3 (lesser curvature), 1 (right cardia), 2 (left cardia) and 7 (left gastric artery) were most frequently involved in type II junctional cancers. The study of Hosokawa *et al*<sup>[40]</sup> came to a similar conclusion. The present study discovered that the perigastric lymph nodes (in Groups 3, 1, 4 and 2) in patients with the cardia cancer ranked the top four positions by metastasis rate, suggesting that the cardiac lymph node is a key dissection object in the reasonable radical operation.

Even after a precise anatomical-topographical differentiation of this tumor entity, Siewert *et al*<sup>[20]</sup> found a small number of patients with parapyloric node metastasis in their cohort with type II adenocarcinoma. Consistent with their finding, in our patient series we found 4.1% of patients with suprapyloric node metastasis and 3.4% with infrapyloric node metastasis. Wang *et al*<sup>[48]</sup> reported that the pathological examination after total gastrectomy showed metastasis rates of lymph nodes in No. 5 and No. 6 of 9.1%-13.6%. They believed that it was difficult to remove all tumor tissues (including metastatic lymph nodes) without total gastrectomy.

Yamashita *et al*<sup>[34]</sup> clearly indicated that dissection of the paracardial and lesser curve lymph nodes offered significant therapeutic benefit, suggesting that these lymph nodes were possibly peritumoral. Furthermore, the number of metastatic nodes in these stations and the total number of metastatic nodes in all stations were equally predictive of the clinical outcome. Dissection of other perigastric nodes, such as Nos. 4sb, 4d, 5, and 6, offered only marginal therapeutic benefit as determined by calculating the index of estimated benefit of nodal dissection. Thus, involvement of the lymph nodes in these stations appeared to represent distant rather than locoregional metastasis<sup>[34]</sup>. Therefore, both esophagectomy with gastric tube reconstruction and gastrectomy with Roux-en-Y reconstruction seem to be valid procedures clinically.

Most series report 7%-40% of mediastinal nodal involvement for type II and III esophagogastric cancer even though abdominal nodes are more affected<sup>[49]</sup>. In our series, mediastinal lymph nodes were affected only in a small number (No. 110, 0.7%), lower than that reported in the literature<sup>[40,49,50]</sup>. The necessity of a prophylactic mediastinal nodal dissection remains controversial. Mine *et al*<sup>[47]</sup> suggested that lower mediastinal lymph nodes,

and station numbers 16A2lat (left renal vein), 11 (splenic artery) and 9 (celiac axis) were the second most frequently involved, and positivity here influenced survival. Hiroharu's data<sup>[34]</sup> suggested that extensive mediastinal lymph node dissection *via* thoracotomy offers no survival benefit over para-periesophageal node clearance alone by the transhiatal approach, which is associated with a lower morbidity, consistent with Sasako' and Hulscher' finding<sup>[51,52]</sup>. Phase III trials in The Netherlands (Dutch trial) and Japan (JCOG 9502) also suggested that an extended transthoracic resection was more hazardous surgery, in terms of morbidity, than a transhiatal esophagectomy. Extended surgery could not be recommended for patients with type II tumors<sup>[52]</sup>. In addition, nodal recurrence was the most frequent in the para-aortic nodes, and less frequent in the mediastinal nodes in Hiroharu's series<sup>[34]</sup>. These results mostly consistent with another report<sup>[40]</sup> support the hypothesis that complete mediastinal nodal clearance is not essential for local control of this disease. Nevertheless, Reeh *et al*<sup>[53]</sup> showed that the presence of lower mediastinal lymph nodes in AOG(oesophago-gastric junction) type II suggests that at least a lower mediastinal dissection should be performed.

Lymph node Nos. 10 and 11 (splenic hilum and splenic artery) belong to pN<sub>2</sub> cardia cancer and have a higher metastatic rate. The high risk factors include female sex, Borrmann type IV, tumor size > 5 cm, poorly differentiated adenocarcinoma, signet ring cell carcinoma, Lauren's diffuse type, vascular lymphatic invasion, and perineural invasion. Some authors believe that a splenectomy must be included for patients with the above high-risk factors<sup>[54]</sup>. Okajima *et al*<sup>[55]</sup> reported that the metastasis rates for lymph node Nos. 10 and 11 in cardia cancer were 15.5% and 12.1%, respectively, and Sakaguchi *et al*<sup>[56]</sup> estimated the rate at 24%. This reflects the status of lymph node metastasis; however, these data were derived from the pathological examination of surgical specimens, mostly based on the corresponding radical operation, and was subject to the understanding of radical surgical indications. Sakaguchi *et al*<sup>[56]</sup> believed that the lymph node metastasis of Nos. 10 and 11 in a larger tumor (> 4 cm), with deeper lesions (T3 and T4) and infiltrative lesions occurred easily. Thus, these clinical characteristics may provide a reference for understanding the indications for combined splenectomy. In our study, the metastasis rates for lymph node No. 10 lymph 1.4%, and for No. 11 it was 2.7%, which are lower than those reported in the literature<sup>[59,40,57]</sup>. Metastasis in these lymph nodes was mainly observed in advanced GC. Therefore, it would be prudent to select the combined resection of distal pancreatectomy and splenectomy for lymph node dissection in patients with cardia cancer<sup>[58,59]</sup>.

The present study showed that the most common sites of the pN<sub>1</sub> lymph node metastasis were Nos. 1-4; the most common sites of the pN<sub>2</sub> lymph nodes were Nos. 7-9; and the most common sites of the pN<sub>3</sub> lymph nodes were Nos. 5 and 6. Lymph node metastasis occurred mostly in the abdominal cavity and lymph node

metastasis of cardia cancer is more similar to that seen for GC. The lymph node metastasis in cardia cancer observed in this study suggests that: (1) for lymph nodes Nos. 1-9, conditions must be focally examined in preoperative ultrasound endoscopic and computed tomography examination; and (2) the superior paragastric fatty tissues should be thoroughly removed in the radical operation for GC and the total lymph node should be dissected in the regions; the celiac trunk and common hepatic artery must be skeletonized and the left gastric artery must be cut to remove the Nos. 7-9 lymph nodes thoroughly.

One analysis showed that 32.9% of type II tumors had involvement of the lymph nodes along the major branched arteries (the left gastric artery, common hepatic artery, splenic artery and celiac axis), and the rate was 50% in type III tumors<sup>[60]</sup>. Siewert *et al*<sup>[61]</sup> also reported similar results; 25% nodal involvement in type II tumors and 39% in type III tumors. These reports clearly indicate that abdominal nodal metastases are frequently observed in adenocarcinoma of the esophagogastric junction type II/III tumors, as in true gastric cancer. Therefore, the extent of a nodal dissection for AEG type II/III should be same as that applied for GC, and an abdominal D2 lymphadenectomy is recommended for patients with type II/III tumors, unless D2 increases the surgical risk<sup>[32]</sup>. Siewert types II and III cancers could be removed safely with an abdominal approach<sup>[45]</sup>. Our results agree with the conclusion of Husemann, that carcinoma of the cardia is a type of carcinoma of the stomach that must be treated according to the criteria of GC surgery<sup>[50]</sup>.

In our study, of 104 patients with lymph node metastases, all were N1, 23 were N2, and 15 were N3. Investigating the correlation between pN<sub>1</sub>, pN<sub>2</sub> and pN<sub>3</sub> lymph node metastases and clinicopathological factors, we found that tumor size, gross appearance, differentiation, pathological depth and lymphatic invasion were associated with lymph node metastases in all patients and male patients at pN<sub>1</sub>. There was an obvious correlation between lymph node metastases and lymphatic invasion in all patients at pN<sub>2</sub>. Univariate analysis of variance revealed a close relationship between lymph node metastases and lymphatic invasion and neural invasion in all patients and lymphatic invasion in female patients at pN<sub>3</sub>. Study of Di Leo *et al*<sup>[10]</sup> study of the treatment of advanced gastric cancer showed that T2 tumors were consistently associated with pN<sub>2</sub> stations nodal infiltration. Such behavior, although less frequent than in T3/4 tumors, does not allow conservative surgery in terms of nodal resection<sup>[10]</sup>.

There were limitations to the present study. First, it was a retrospective study based on postoperative examination of resected specimens. Second, the number of patients was low. Thus, further study with a larger sample size should be carried out to confirm our results. Otherwise, the extent of nodal involvement was most likely underestimated. The lack of information of nodal status at specific remote sites in some cases also made the investigation of nodal stage migration impossible. The retrospective nature of this study meant that there was

some selection bias, such as the surgeon's preference for a thoracoabdominal or transabdominal approach.

In conclusion, the findings in this study indicate that the clinicopathological features and risk factors for lymph node metastasis of male and female patients with gastric cardia adenocarcinoma did not differ significantly. Therefore, the effect of male sex on the clinical course of gastric cardia adenocarcinoma had a weak impact in comparison to female sex once a curative resection had been performed. However, further evaluations should be performed. The outcome should improve if male patients, as well as female patients, undergo careful diagnosis of malignancy and early multimodality treatment.

## COMMENTS

### Background

As gastric cancer incidence declines, the frequency of proximal gastric and gastroesophageal junctional adenocarcinomas continues to rise, and has become a significant clinical challenge. Adenocarcinoma of the cardia generally has a low curative resection rate and a poor prognosis; worse than carcinoma of other regions of the stomach, mainly because the disease is at a more advanced stage at diagnosis. It is crucial that the therapeutic strategy for gastric cardia adenocarcinoma be clarified through evaluation of both the pattern of lymph node metastasis and the efficacy of lymph node dissection in this region.

### Research frontiers

The optimal surgical strategy for tumors in the cardia area of the stomach, especially tumors invading the lower esophagus, remains controversial. The development of effective therapeutic strategies for these tumors requires information on patient characteristics, patterns of lymph node metastasis, and the efficacy of lymph node dissection.

### Innovations and breakthroughs

Univariate analysis showed an obvious correlation between lymph node metastases and tumor size, gross appearance, pathological depth and lymphatic invasion in male patients. Multivariate logistic regression analysis revealed that tumor differentiation and pathological depth were independent risk factors for lymph node metastases in male patients. There was an obvious relationship between lymph node metastases and tumor size, gross appearance, differentiation, pathological depth, lymphatic invasion at pN<sub>1</sub>, and lymphatic invasion at pN<sub>2</sub> and neural invasion at pN<sub>3</sub> in male patients. There were no significant differences in clinicopathological features or lymph node metastases between female and male patients.

### Applications

Tumor differentiation and depth were risk factors for lymph node metastases in male patients with gastric cardia adenocarcinoma and should be considered when choosing surgery.

### Terminology

The definition of the cardia commonly employed in Japan is the area within 2 cm above and below the esophagogastric junction, and tumors whose center is situated in this area are considered to be cancer of the cardia; such cancers are distinguished from upper gastric cancers. Siewert and Stein proposed a topographical classification for cardia carcinomas.

### Peer review

Congratulate the authors for an excellent effort. As rightly highlighted, a future attempt in expanding the population size should hopefully provide further insights.

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## Research on stress-induced apoptosis of natural killer cells and the alteration of their killing activity in mouse liver

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### Abstract

**AIM:** To investigate the stress-induced apoptosis of natural killer (NK) cells and the changes in their killing activity in mouse livers.

**METHODS:** A restraint stress model was established in mice. Flow cytometry was employed to measure the percentage of NK cells and the changes in their absolute number in mouse liver. The cytotoxicity of hepatic and splenic NK cells was assessed against YAC-1 target cells *via* a 4 h <sup>51</sup>Cr-release assay.

**RESULTS:** The restraint stress stimulation induced the apoptosis of NK cells in the liver and the spleen, which decreased the cell number. The number and percentage of NK cells in the spleen decreased. However, the number of NK cells in the liver decreased, whereas the percentage of NK cells was significantly increased. The apoptosis of NK cells increased gradually with prolonged stress time, and the macrophage-1 (Mac-1)<sup>+</sup> NK cells were more susceptible to apoptosis than Mac-1<sup>-</sup> NK cells. Large numbers of Mac-1<sup>-</sup> NK cells in the liver, which are more resistant to stress-induced apoptosis, were observed than the Mac-1<sup>-</sup> NK cells in the spleen. The stress stimulation diminished the killing activity of NK cells in the spleen was significantly decreased, but the retention of numerous Mac-1<sup>-</sup> NK cells in the liver maintained the killing ability.

**CONCLUSION:** Significant stress-induced apoptosis was observed among Mac-1<sup>+</sup> NK cells, but not Mac-1<sup>-</sup> NK cells in the mouse liver. Stress stimulation markedly decreased the killing activity of NK cells in the spleen but remained unchanged in the liver.

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**Key words:** Restraint stress; Natural killer cells; Cell apoptosis; Killing activity

**Core tip:** Hepatic natural killer (NK) cells are classified into macrophage-1 (Mac-1)<sup>+</sup> and Mac-1<sup>-</sup> cells, and the different functional characteristics of Mac-1<sup>+</sup> or Mac-1<sup>-</sup> NK cells in response to stress stimulation are confirmed. This study further proves the heterogeneity of NK cell function, and the results provide a reference for preventing the immune system damage caused by stress.

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## INTRODUCTION

In the modern society, the acceleration of work and life style and the deterioration of the environment, as well as natural disasters and frequent traffic accidents, expose people to increasing stress. Prolonged or intense stress that overwhelms autoregulation, causes nervous, endocrine, and immune system dysfunction<sup>[1-3]</sup>, as well as the apoptosis of lymphocytes such as natural killer (NK) cells, B cells and T cells. Immune system dysfunction is the direct cause of infectious diseases, cancers and self-deterioration<sup>[4-6]</sup>.

NK cells are an important type of lymphocytes, accounting for 10% to 15% of total lymphocytes and play a crucial role in body resistance to against infections and tumours, as well as immune and hematopoietic regulation<sup>[7,8]</sup>.

This study aims to determine the effects of persistent and intense stress on the apoptosis of hepatic and splenic NK cells and change in their function in mice.

## MATERIALS AND METHODS

### Experimental animals

Eight-week-old clean grade C57BL/6 mice were purchased from the Animal Centre of The 3<sup>rd</sup> Affiliated Hospital of Harbin Medical University.

### Mouse model preparation

The protocol used has been described in the published literature<sup>[9]</sup>. The mice were placed in a 50 mL Falcon tube with 4 to 5 drilled holes at the bottom to maintain ventilation. Sufficient amounts of absorbent cotton were placed inside the tube to immobilise the mouse, and then the lid was screwed shut. The tube was kept at room temperature for 24 h, and the mouse was not fed any food and water. The mice in the control group were left in the original cage without any disturbance.

### Mouse lymphocyte preparation

The protocol used has been described in the published literature<sup>[10]</sup>. The mouse was anaesthetised with ether and sacrificed *via* heart puncture. Subsequently, the mouse liver and spleen were collected and minced. The tissue sample was washed with phosphate-buffered saline (PBS) and filtered with 200 mesh strainer, and then the cell suspension was collected. After gradient centrifugation, the cells were lysed with 0.83% NH<sub>4</sub>Cl-Tris buffer (pH 7.6). The resulting cell suspension was collected and the concentration was adjusted to  $1.0 \times 10^6$ /mL.

### Immunofluorescence labelling

Lymphocytes were isolated from mouse liver and spleen, and then double or triple immunofluorescence staining was performed to identify the CD<sub>3</sub><sup>+</sup>NK<sub>1.1</sub><sup>+</sup> cells as NK

cells. Fluorescein-isothiocyanate (FITC)-labelled antibodies: CD<sub>3</sub> (145-2C11 clone); PE-labelled antibody: NK<sub>1.1</sub> (PK136 clone); Biotin-labelled antibody: macrophage-1 (Mac-1) (M1/70 clone) CD<sub>69</sub> (H1.2F3 clone), Ly49C/I (5E6 clone). All the monoclonal antibodies were purchased from BD Biosciences Pharmingen in San Diego, United States.

Cell suspension was transferred in centrifuge tube (cell number  $< 2 \times 10^6$ ). After 2 min of centrifugation at 2500 r/min and 4 °C, the supernatant was removed, followed by vibration. Then, 10 μL of 2.4 G2 was added (anti-FcγR II / III). After incubation at 4 °C for 10 min, 10 μL of various monoclonal antibodies (CD<sub>3</sub>, NK<sub>1.1</sub>, Mac-1, CD<sub>69</sub>, and Ly49C) were added, accordingly. After vortex and incubation at 4 °C for 20 min, the cells were washed once with PBS (2500 r/min at 4 °C). For double staining, the cells were diluted with 0.5 mL of PBS and filtered with nylon mesh, and then 5 μL of propidium iodide (PI) was added for flow cytometry analysis. When subjected for triple staining, cells were incubated with 10 μL of biotin-labelled secondary antibody at 4 °C for 20 min, and then washed with PBS once (2500 r/min at 4 °C). After diluting with 0.5 mL of PBS and filtration with a nylon mesh, the stained cells were analyzed *via* flow cytometry<sup>[11]</sup>. Flow Cytometer was FAC sort from BD-United States and software was Cell Quest 3.0.

### Detection of killing activity of NK cells

The cytotoxicity of NK cells was assessed against YAC-1 target cells. Target cells were continuously cultured for 24 h in RPMI 1640 containing 200 mL/L FCS. YAC-1 cells were collected at exponential phase and counted through trypan blue staining. Viable cells were considered as targets cells when their percentage exceeded 95%. The cell concentration was adjusted to  $1 \times 10^5$  /mL with RPMI 1640. After incubation with <sup>51</sup>Cr for 2 h, the cells were washed three times with RPMI 1640 to remove free <sup>51</sup>Cr. The target cell concentration was adjusted to  $1.0 \times 10^4$ /mL or  $2.0 \times 10^4$ /mL. The cells were divided into three groups: NK cell group, target cell maximum release group, and target cell spontaneous release group. Subsequently, the cells were seeded in U-bottom microplates (96-well). Lymphocytes in the mouse liver and spleen were utilized as effector cells, which were added into the U-bottom microplates at an effector:target ratio of 50:1, 25:1, and 12.5:1 in a volume of 100 μL/well. The cells were incubated at 37 °C with 5% CO<sub>2</sub> for 4 h and the microplates were centrifuged at 1500 r/min for 5 min. About 100 μL of supernatant was collected from each well, and its radioactivity (CPM) was measured with gamma counter<sup>[12]</sup>.

The specific killing rate was calculated using the following formula: specific killing rate (%) = (experimental cell release - target cell spontaneous release)/(target cell maximum release - target cell spontaneous release) × 100%.

### Statistical analysis

The data are presented as mean ± SD and percentage.

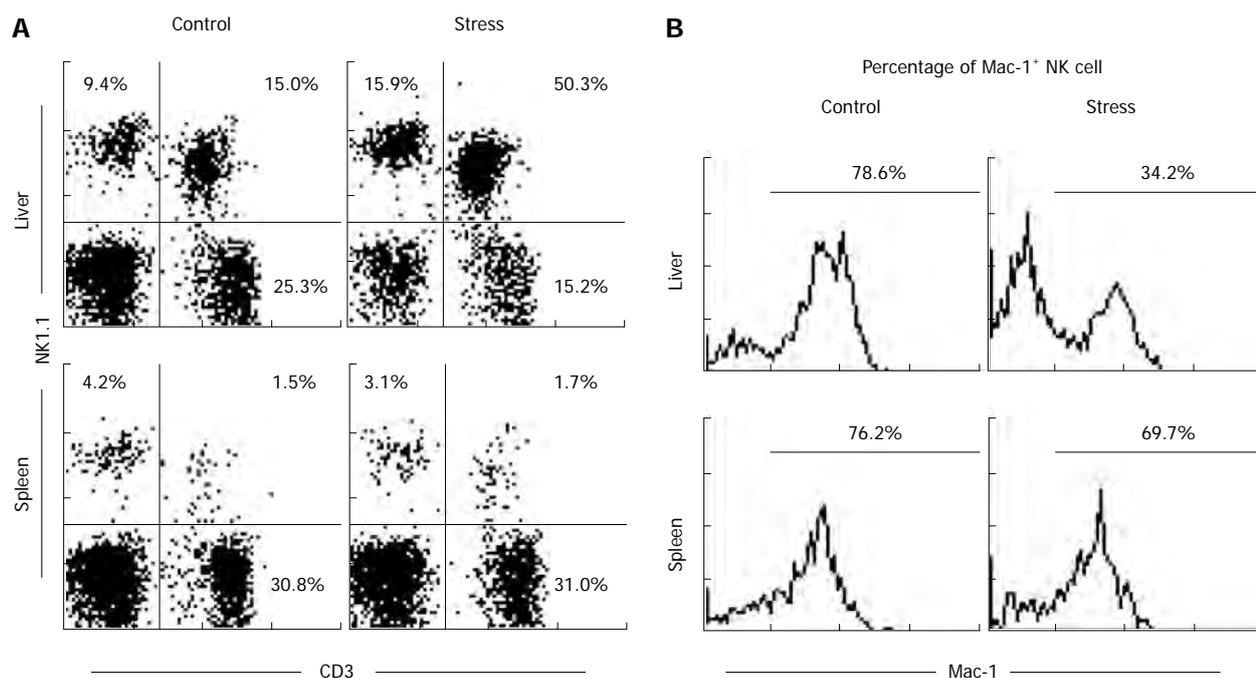


Figure 1 Percentage of natural killer cells and macrophage-1<sup>+</sup> natural killer cells in mouse liver and spleen after 24 h of restraint stress. A: Natural killer cells; B: Macrophage-1<sup>+</sup> natural killer cells. NK: Natural killer; Mac-1: Macrophage-1.

Significant differences between two samples were analyzed with Student's *t* test.

## RESULTS

### Stress stimulation and the change of NK cell number

The percentage of splenic NK cells in total lymphocytes did not change significantly ( $3.9\% \pm 1.2\%$  vs  $2.6\% \pm 1.1\%$ ,  $P > 0.05$ ), whereas the percentage of hepatic NK cells increased ( $8.6\% \pm 1.3\%$  vs  $14.9\% \pm 1.5\%$ ,  $P < 0.05$ ; Figure 1).

After restraint stress stimulation, the numbers of lymphocytes in the experimental and control groups were  $(53.1 \pm 9.7) \times 10^5$  vs  $(19.7 \pm 4.6) \times 10^5$  ( $n = 6$ ,  $P < 0.05$ ) in liver,  $(87.7 \pm 9.6) \times 10^6$  vs  $(36.4 \pm 7.1) \times 10^6$  ( $n = 6$ ,  $P < 0.05$ ) in spleen. The number of NK cells in the experimental and control groups were  $(57.4 \pm 8.9) \times 10^5$  vs  $(24.6 \pm 7.3) \times 10^5$  ( $n = 6$ ,  $P < 0.05$ ) in the liver,  $(29.7 \pm 6.5) \times 10^6$  vs  $(8.6 \pm 1.4) \times 10^6$  ( $n = 6$ ,  $P < 0.05$ ) in the spleen (Figure 2).

### Number of Mac-1<sup>+</sup> and Mac-1<sup>-</sup> NK cells

NK cells were isolated from mouse liver and spleen after 24 h of restraint stress stimulation, followed by immunofluorescence staining with FITC: Mac-1 and PE: NK1.1. The cells were analyzed *via* flow cytometry. The results show that the percentage of hepatic Mac-1<sup>+</sup> NK cells in the experimental group was significantly higher than that of the control group ( $77.2\% \pm 1.7\%$  vs  $33.9\% \pm 1.1\%$ ,  $P < 0.05$ , Figure 1B), whereas the percentage of hepatic Mac-1<sup>-</sup> NK cells relatively increased. The percentage of splenic Mac-1<sup>+</sup> NK cells was slightly decreased ( $75.1\% \pm 1.1\%$  vs  $68.5\% \pm 1.6\%$ ,  $P > 0.05$ ).

After 24 h of stress stimulation, the number of Mac-1<sup>+</sup> NK cells in the liver and spleen in the stress group was significantly lower than those in the control group: liver,  $(37.7 \pm 9.8) \times 10^4$  vs  $(8.4 \pm 1.7) \times 10^4$  ( $n = 6$ ,  $P < 0.05$ ); spleen,  $(23.5 \pm 6.3) \times 10^5$  vs  $(8.7 \pm 1.9) \times 10^5$  ( $n = 6$ ,  $P < 0.05$ ). The results are shown in Figure 3.

### Stress stimulation and apoptosis of NK cells

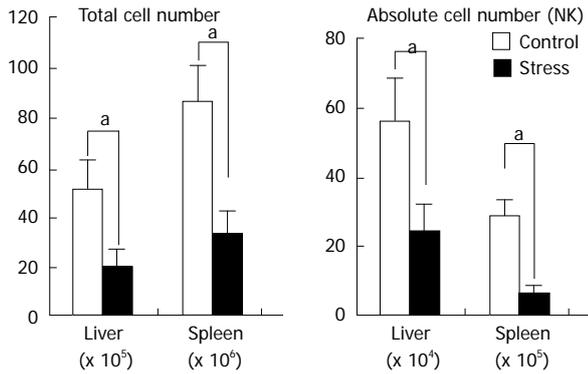
NK cells were isolated from mouse liver after 24 h of restraint stress stimulation, followed by immunofluorescence staining with Annexin V-FITC and PI. The cells were analyzed *via* flow cytometry. The results revealed significant apoptosis of NK cells ( $9.5\% \pm 1.4\%$  vs  $19.3\% \pm 1.3\%$ ,  $P < 0.05$ ), especially the Mac-1<sup>+</sup> NK cells ( $5.2\% \pm 1.8\%$  vs  $19.3\% \pm 1.4\%$ ,  $P < 0.05$ ), whereas the apoptosis of Mac-1<sup>-</sup> NK cells did not significantly change ( $13.4\% \pm 1.3\%$  vs  $7.4\% \pm 1.7\%$ ,  $P > 0.05$ ; Figure 4).

### Stress stimulation and the change of killing activity mediated by NK cells

The killing activity of NK cells was markedly decreased in the spleen ( $16.7\% \pm 1.4\%$  vs  $8.9\% \pm 1.1\%$ ,  $P < 0.05$ ), but was sustained in the liver even after 24 h of restraint stress stimulation (Figure 5).

## DISCUSSION

With the development of modern medical technology, increasing attention has been focused on the relationship between stress and health. The response of the body to stress is a dynamic balance, which allows the body to recover to its original state through autoregulation after stress reaction. However, persistent stress may overwhelm



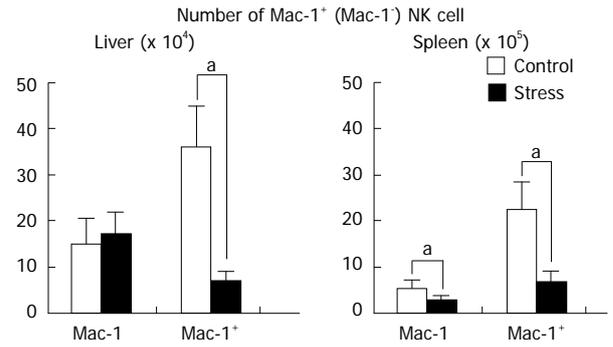
**Figure 2** The number of lymphocytes and of natural killer cells in mouse liver and spleen after 24 h of restraint stress. NK: Natural killer. <sup>a</sup>*P* < 0.05 vs control group.

autoregulation and cause psychosomatic damage<sup>[13,14]</sup>.

Numerous studies have demonstrated that acute stress stimulation causes distinctly reduced number of lymphocytes in the thymus, spleen, peripheral blood, and liver, as well as disrupt the function of T, B and NK cells. Moreover, the decrease in lymphocytes mainly results from apoptosis.

Our research shows that the number of splenic lymphocytes was significantly decreased, but their percentage did not change significantly. We speculate that various lymphocytes in spleen decreased with the same percentage, which is consistent with the results of previous studies<sup>[10,15]</sup>. Moreover, the results showed that the number of hepatic lymphocytes significantly declined but the percentage of NK cells dramatically increased (Figures 1A and 2). Therefore, the number of NK cells in the liver and the spleen were determined. Although both the liver and the spleen had fewer NK cells, the number of NK cells was relatively high in the liver, dramatically increasing the percentage of NK cells in the liver (Figure 2). These results indicate that the hepatic NK cells differed from splenic NK cells after stress stimulation. A recent study reported that NK cells have organ specificity, which allows liver to be considered as immune organ and NK cells possess unique functional characteristics<sup>[16,17]</sup>.

Mac-1 was employed to distinguish the subtypes of NK cells with different functions. Mac-1 (CD11b/CD18) is an adhesion molecule of the integrin family, highly expressed in most myeloid hematopoietic cells, such as neutrophils, monocytes/macrophages, eosinophils and B cells. Mac-1 is closely correlated with cell phagocytosis and adhesion, as well as a marker for myeloid and lymphoid hematopoietic cells. NK cells are the only lymphoid cells that express Mac-1. Some researchers have considered Mac-1 as a marker for mature NK cells, which have cell killing activity and are able to secrete cytokines, whereas Mac-1<sup>-</sup> NK cells are immature, with limited cell killing activity and cytokine production<sup>[18-20]</sup>. Mac-1 is expressed by 80% to 90% of mature NK cells in the liver, spleen, and peripheral blood. Therefore, current research on NK cells is mainly focused on Mac-1<sup>+</sup> NK cells. Our previous study discovered numerous Mac-1<sup>-</sup> NK cells



**Figure 3** Determination of macrophage-1<sup>+</sup> and macrophage-1<sup>-</sup> natural killer cells in mouse liver and spleen after 24 h of restraint stress. Mac-1<sup>+</sup>: Macrophage-1 positive; Mac-1<sup>-</sup>: Macrophage-1 negative; NK: Natural killer. <sup>a</sup>*P* < 0.05 vs control group.

in the liver and demonstrated that Mac-1<sup>+</sup> NK cells and Mac-1<sup>-</sup> NK cells have different functional characteristics and cell phenotypes<sup>[11]</sup>. Therefore, hepatic NK cells were classified into Mac-1<sup>+</sup> and Mac-1<sup>-</sup> subtypes according to Mac-1 expression.

Our study shows that the number of Mac-1<sup>+</sup> and Mac-1<sup>-</sup> NK cells in the spleen decreases with same percentage after stress stimulation, which results in sustained Mac-1 expression in splenic NK cells. The number of hepatic Mac-1<sup>+</sup> NK cells significantly decreased, whereas the number of Mac-1<sup>-</sup> NK cells did not change significantly, which accounts for the reduced Mac-1 expression in hepatic NK cells (Figures 1B and 3). Therefore, we speculate that Mac-1<sup>+</sup> hepatic NK cells are resistant to the apoptosis induced by stress stimulation.

A large number of studies have demonstrated that acute stress stimulation causes distinctly reduced number of lymphocytes in the thymus, spleen, peripheral blood, and liver, as well as disrupts the function of T, B and NK cells. Moreover, the decrease in lymphocytes is mainly caused by the apoptosis of lymphocytes.

Intracellular Annexin V expression was measured to assess the degree of apoptosis of hepatic NK cells induced by stress stimulation<sup>[21]</sup>. The results revealed significant NK cell apoptosis, especially Mac-1<sup>+</sup> NK cells, whereas Mac-1<sup>-</sup> NK cell apoptosis remained unchanged (Figure 4).

To determine the effects of stress on NK cell function, we employed YAC-1 as target cells to measure killing activity of NK cells. The results imply that stress stimulation decreases the killing activity of splenic NK cells, which accords with the results of previous research. Our study also discovered that stress stimulation does not affect the killing activity of hepatic NK cells, which contrasts with the conclusion of previous investigations. We concluded that Mac-1<sup>+</sup> NK cells had stronger killing activity than that of Mac-1<sup>-</sup> NK cells<sup>[11]</sup>. We also believed that the number of hepatic Mac-1<sup>+</sup> NK cells declined because of cell apoptosis, which allowed apoptosis-resistant Mac-1<sup>-</sup> NK cells to survive, and to exhibit relatively strong cell killing activity.

In conclusion, our research preliminarily demon-

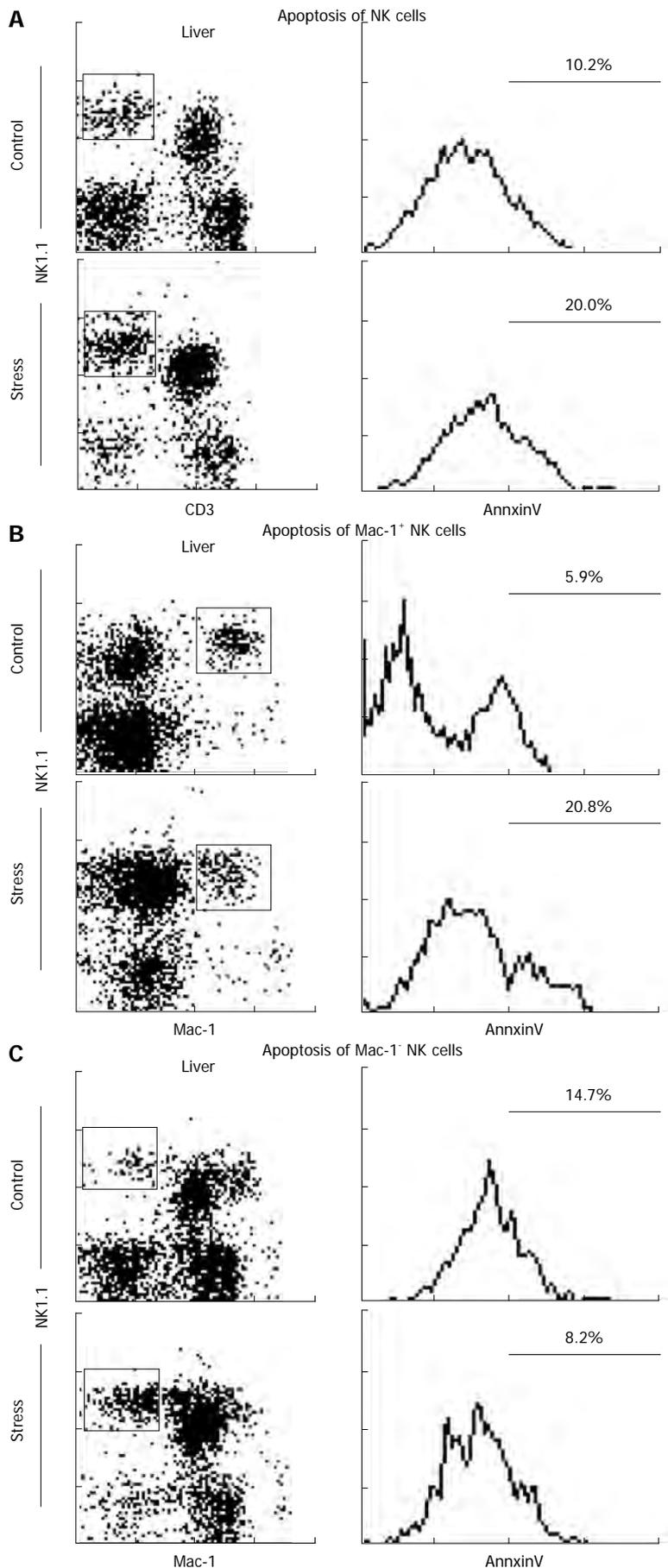


Figure 4 Apoptosis of natural killer cells after 24 h of restraint stress. A: Apoptosis of NK cells; B: Apoptosis of Mac-1<sup>+</sup> NK cells; C: Apoptosis of Mac-1<sup>-</sup> NK cells. NK: Natural killer; Mac-1: Macrophage-1.

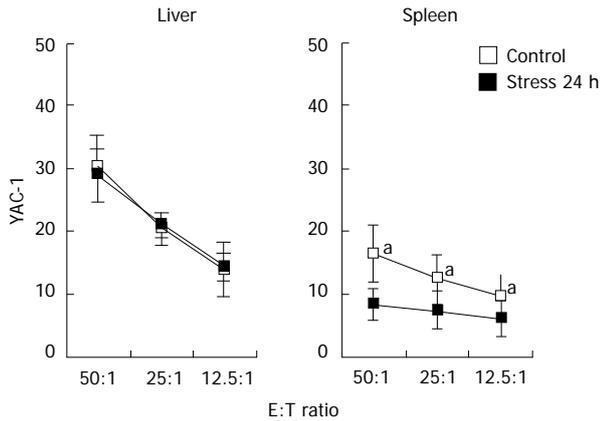


Figure 5 Cytotoxicity of natural killer cells in mouse liver and spleen after 24 h of restraint stress. NK: Natural killer; E:T Ratio: Effector-target ratio. <sup>a</sup> $P < 0.05$  vs spleen after 24 h of stress.

states that intense stress stimulation induces the apoptosis of Mac-1<sup>+</sup> hepatic NK cells instead of Mac-1<sup>-</sup> NK cells, which requires further investigation to understand the underlying mechanisms and the role of the liver in stress-triggered immune function.

## COMMENTS

### Background

Stress refers to non-specific systemic reactions to strong stimulus on the body. Intense and prolonged stress stimulation causes immune function disorders, volume shrinkage, and dysfunction of immune organs such as the liver, spleen, and thymus gland. In addition, stress stimulation causes the apoptosis and dysfunction of lymphocytes such as natural killer (NK), T, and B cells in the peripheral blood and immune organs. Most studies have demonstrated that strong stress stimulation may decrease NK cell killing activity in the peripheral blood and spleen, which weakens the immune system.

### Research frontiers

NK cells are a class of lymphocytes in the innate immune system that account for about 10% to 15% of all lymphocytes. They have anti-infective, anti-tumour, and immunomodulatory functions, and they regulate hematopoiesis. NK cells mainly function in killing cells and cytokine secretion. NK cells are divided into two subtypes according to surface antigens and functional cell expression, namely, NK<sub>1</sub> and NK<sub>2</sub>. Previous investigations have studied NK cells in the peripheral blood and the spleen. The results confirm that the liver generates NK cells during embryogenesis and the function of NK cells in the liver is different from that in the peripheral blood and the spleen.

### Innovations and breakthroughs

This study confirms that stress stimulation significantly decreases the number of splenic NK cells, with significantly decreased killing activity, whereas some NK cells survive in the liver. Further research proves that these surviving cells are macrophage-1 (Mac-1<sup>-</sup>) NK cells that resist stress-induced cell apoptosis. By contrast, the killing activity of Mac-1<sup>+</sup> NK cells is unaffected by stress stimulation.

### Applications

This study proves the anti-stress ability of Mac-1<sup>-</sup> hepatic NK cells. This finding suggests that Mac-1<sup>-</sup> NK cells maintain immune functional stability under stress conditions. Further studies should investigate how to characterize Mac-1<sup>-</sup> NK cells and utilize them for preventing the immune dysfunction caused by stress.

### Terminology

NK cells are important immune cells with anti-tumour, anti-viral, and immune regulation function, but also participate in the hypersensitivity and occurrence of autoimmune diseases in some cases. Mac-1 (CD11b/CD18) is an adhesion molecule (integrins), and is expressed in most myeloid hematopoietic cells such as neutrophils, monocyte-macrophages, eosinophils, and B cells.

## Peer review

Authors investigated the stress-induced apoptosis of NK cells and the changes in their killing activity in mouse livers. NK cell is an important type of lymphocytes. The authors made an interesting research on NK cell. This study proves the anti-stress ability of Mac-1<sup>-</sup> hepatic NK cells. This finding suggests that Mac-1<sup>-</sup> NK cells maintain immune functional stability under stress conditions. Further studies should investigate how to characterize Mac-1<sup>-</sup> NK cells and utilize them for preventing the immune dysfunction caused by stress.

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## Interaction between cyclooxygenase-2, Snail, and E-cadherin in gastric cancer cells

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Author contributions: Zhou YN and Qiao L designed the research; Liu XJ, Chen ZF, Li HL, Tian AP, Hu ZN and Liu M performed the laboratory studies; Wu J and Zhao D analyzed the data; Liu XJ and Chen ZF wrote the paper.

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### Abstract

**AIM:** To investigate the mechanisms of how cyclooxygenase-2 (COX-2) regulates E-cadherin in gastric cancer cells.

**METHODS:** COX-2 expression in human gastric cancer cell lines SGC-7901, BGC-823, MGC-803 and AGS were measured at the mRNA and protein level. COX-2 rich cell line SGC-7901 was chosen for subsequent experiments. siRNA mediated gene knockdown was used to investigate the impact of COX-2 on nuclear factor- $\kappa$ B

(NF- $\kappa$ B), Snail, and E-cadherin in gastric cancer cells. Gene expression was determined by Western blot and real-time polymerase chain reaction. To analyze whether NF- $\kappa$ B inhibition could interrupt the modulatory effect of COX-2 or prostaglandin E2 (PGE2) on E-cadherin, gastric cancer cells were treated with celecoxib or PGE2, in the presence of NF- $\kappa$ B specific siRNA.

**RESULTS:** Highest expression level of COX-2 was found in SGC-7901 cells, both at mRNA and protein levels. siRNA mediated down-regulation of COX-2 led to a reduced expression of NF- $\kappa$ B and Snail, but an increased expression of E-cadherin in SGC-7901 cells. siRNA mediated down-regulation of NF- $\kappa$ B also led to a reduced expression of E-cadherin and Snail in SGC-7901 cells. However, COX-2 expression did not alter after cells were treated with NF- $\kappa$ B specific siRNA in SGC-7901 cells. Treatment of SGC-7901 cells with celecoxib led to a reduced expression of Snail but an increased expression of E-cadherin. In contrast, treatment of SGC-7901 cells with PGE2 led to an increased Snail and a decreased E-cadherin. However, siRNA-mediated knockdown of NF- $\kappa$ B partially abolished the effect of celecoxib and PGE2 on the regulation of E-cadherin and Snail in SGC-7901 cells.

**CONCLUSION:** COX-2 likely functions upstream of NF- $\kappa$ B and regulates the expression of E-cadherin *via* NF- $\kappa$ B/Snail signaling pathway in gastric cancer cells.

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**Key words:** Cyclooxygenase-2; E-cadherin; celecoxib; Prostaglandin E2; Gastric cancer

**Core tip:** Cyclooxygenase-2 (COX-2) plays an important role in transcriptional regulation of E-cadherin in gastric cancer and other malignancies. On the contrary, prostaglandin E2 (PGE2) promotes invasion of tumor cells through down-regulating the expression of E-cadherin.

Our study has provided further evidence that COX-2 functions upstream of nuclear factor- $\kappa$ B in the regulation of Snail and E-cadherin in gastric cancer cells. Blockade of COX-2 activity or inhibition of PGE2 production may offer some benefit in the chemoprevention and treatment of gastric cancer.

Liu XJ, Chen ZF, Li HL, Hu ZN, Liu M, Tian AP, Zhao D, Wu J, Zhou YN, Qiao L. Interaction between cyclooxygenase-2, Snail, and E-cadherin in gastric cancer cells. *World J Gastroenterol* 2013; 19(37): 6265-6271 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i37/6265.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6265>

## INTRODUCTION

Cyclooxygenase-2 (COX-2) is an inducible isozyme of cyclooxygenase and catalyzes prostaglandin E2 (PGE2) formation in response to various inflammatory stimuli or growth factors<sup>[1]</sup>. PGE2 plays an important role in regulating diverse cellular functions under physiological and pathological conditions<sup>[2]</sup>. Overexpression of COX-2 is related to invasion and metastasis of tumor cells<sup>[3-5]</sup>. To further support the role of COX-2 in tumor promotion, it was reported that PGE2 was able to facilitate the invasion of tumor cells through down-regulation of E-cadherin<sup>[6]</sup>. On the other hand, celecoxib, a selective inhibitor of COX-2, could inhibit migration and metastasis of tumor cells by up-regulating E-cadherin<sup>[7]</sup>. Many studies have suggested that COX-2 is generally overexpressed in gastric cancer tissues, and it was thought to play a crucial role in the development and invasion of gastric cancers<sup>[8]</sup>. In contrast, the expression of E-cadherin, an important cell adhesion molecule, is usually low, mutated, or even lost in gastric cancer tissues<sup>[9,10]</sup>. Thus, COX-2 and E-cadherin appear to exhibit totally different expression patterns. Our group had previously reported an inverse correlation between COX-2 and E-cadherin and suggested that Snail is likely to be responsible for the regulation of COX-2 on the expression and function of E-cadherin in gastric cancer tissues<sup>[11,12]</sup>.

Snail is a transcription factor and was reported to down-regulate the expression of E-cadherin, causing disruption to cell-to-cell adhesion and thereby facilitates tumor progression and metastases<sup>[13,14]</sup>. Meanwhile, it was reported that nuclear factor- $\kappa$ B (NF- $\kappa$ B) promotes tumor cell migration and invasion in many human cancers through up-regulating Snail and subsequent suppression of E-cadherin<sup>[15-17]</sup>.

Therefore, it is very likely that the interaction between COX-2, Snail, and E-cadherin may play a key regulatory role in invasion and metastasis of gastric cancer. Our group is interested in understanding the possible interaction between COX-2, Snail, and E-cadherin during the development, progression, invasion, and metastasis of gastric cancer. Thus, the aim of this study is to investigate if COX-2 modulates E-cadherin expression *via* Snail and

NF- $\kappa$ B in gastric cancer cells.

## MATERIALS AND METHODS

### Reagents and cell lines

RPMI 1640 medium and PGE2 were purchased from Sigma-Aldrich (St. Louis; MO, United States). Opti-MEM I Reduced Serum Medium, Lipofectamine 2000, BLOCK-iT™ Fluorescent Oligo, and negative control for RNAi were purchased from Invitrogen (Carlsbad, CA, United States). Fetal calf serum was purchased from Hyclone Laboratories (Logan, UT, United States). Reverse transcription kit and quantitative polymerase chain reaction (qPCR) kit were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). celecoxib was purchased from Cayman Chemical (Ann Arbor, MI, United States). Polyclonal antibodies against COX-2, NF- $\kappa$ B p65, E-cadherin, and  $\beta$ -actin were from BioWorld Corporation (CA, United States). Polyclonal antibody against human Snail was purchased from Abcam (Cambridge, United Kingdom). All primers were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). Double strand (ds) RNAi Stealth™ oligos, the specific siRNA against COX-2 and NF- $\kappa$ B (p65) were designed and synthesized by Invitrogen (Carlsbad, CA, United States).

Human gastric cancer cell lines SGC-7901, BGC-823, MGC-803 and AGS were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

### Cell culture

Gastric cancer cell lines (SGC-7901, BGC-823, MGC-803 and AGS) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and maintained at 37 °C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub>. Before transfection, the culture medium RPMI 1640 was replaced by Opti-MEM I.

### Baseline expression of COX-2 in gastric cancer cells

SGC-7901, BGC-823, MGC-803 and AGS were plated respectively at a concentration of 10<sup>5</sup> cells/ well in a 6-well plate and incubated overnight. Total RNA and protein were extracted to determine the basal expression level of COX-2 at the mRNA by PCR and protein level by Western blot, respectively.

### siRNAs design, transient transfection of SGC-7901 cells with COX-2 and NF- $\kappa$ B siRNA oligonucleotides

As the SGC-7901 cells showed the highest expression level of COX-2, we used siRNA knockdown approach to investigate the impact of COX-2 on NF- $\kappa$ B, Snail, and E-cadherin in this cell line. Three pairs of siRNA oligos against COX-2 and NF- $\kappa$ B p65, and a control (scrambled) siRNA were initially designed and commercially synthesized. The sequences of these siRNAs were shown in Table 1.

For transfection, cells were seeded into a 6-well plate at a density of 3 × 10<sup>5</sup> cells per well and incubated overnight. Cells were then transfected with siRNA oligos

**Table 1** Sequences of the specific siRNA against cyclooxygenase-2 and nuclear factor- $\kappa$ B p65 used in the study

siRNA against	Forward	Reverse
COX-2	AAUAGGAGAG- GUUAGAGAAGGCUUC	GAAGSCUUCUCAAC- CUCUCCUAUU
NF- $\kappa$ B p65	UCACUAGGC- GAGUUUAUAGSCUCAGG	CCUGAGGCUAUAA- CUCGCCUAGUGA

COX-2: Cyclooxygenase-2; NF- $\kappa$ B: Nuclear factor- $\kappa$ B.

using Lipofectamine 2000 and incubated for 24 to 72 h before further analysis. Transfection efficiency was determined by transfecting the cells with FITC labeled Oligo and counting the number of positive cells under the fluorescent microscopy. More than 80% of cells were routinely successfully transfected. The expression of COX-2, NF- $\kappa$ B, Snail and E-cadherin were analyzed by qPCR and Western blot in successfully transfected cells.

#### Co-treatment of SGC-7901 cells with NF- $\kappa$ B specific siRNA, celecoxib, and PGE2

To analyze whether NF- $\kappa$ B inhibition could interrupt the modulation effect of COX-2 or PGE2 on E-Cadherin, SGC-7901 cells were treated with 40  $\mu$ mol/L celecoxib for 24 h alone or with NF- $\kappa$ B specific siRNA. Cells were also treated with 10  $\mu$ mol/L PGE2 for 4 h alone or with NF- $\kappa$ B specific siRNA. The optimal dosages for celecoxib and PGE2 were based on our preliminary study. The expression of NF- $\kappa$ B, Snail and E-cadherin were measured by qPCR and Western blot.

#### Western blotting for COX-2, NF- $\kappa$ B, Snail and E-cadherin expression

Whole-cell extracts were prepared from the treated cells with 2 mL of RIPA buffer containing protease inhibitors. Cell lysates were centrifuged at 8000 rpm for 10 min and the supernatant was collected. Cell lysates were electrophoretically separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to nitrocellulose membrane and the membrane was blocked with 5% fat-free milk in TBS plus 0.1% Tween-20 (TBST). The membranes were then incubated with respective primary antibodies (rabbit polyclonal COX-2, NF- $\kappa$ B p65, Snail, E-cadherin, all at 1:1000 dilution) and the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h. The membranes were incubated with enhanced chemiluminescence system and exposed to X-ray film for signal detection.  $\beta$ -actin was used as a control for equal loading of samples.

#### Real-time PCR for COX-2, NF- $\kappa$ B, Snail and E-cadherin expression

Total RNA was extracted with Trizol reagent according to the manufacturer's instructions. Approximately 30 ng of total RNA was transcribed into cDNA. The synthesized cDNA samples were subjected to qPCR using SYBR<sup>®</sup>

**Table 2** The sequences of the primers used in this study

Primers	Sense primer	Antisense primer
COX-2	5'-GCCTGAATGTGCCATA AGACTGAC-3'	5'-AAACCCACAGTGCTTG ACACAGA-3'
E-cadherin	5'-TACTACTGCCAGGAGS CAGA-3'	5'-TGGCACCAGTGTCGG ATTA-3'
Snail	5'-GACCACTATGCCGCGC TCTT-3'	5'-TCGCTGTAGTTAGGCT TCCGATT-3'
NF- $\kappa$ B p65	5'-TCAGTCAGSGCATCCA GACC-3'	5'-CAGAGSCGCACAGSAT TCA-3'
$\beta$ -actin	5'-TGGCACCCAGSACAAT GAA-3'	5'-CTAAGTCATAGTCCGC CTAGAAGSA-3'

COX-2: Cyclooxygenase-2; NF- $\kappa$ B: Nuclear factor- $\kappa$ B.

Green Quantitative PCR kit. Amplification was carried out in a total volume of 20  $\mu$ L for 40 cycles of 15 s at 95  $^{\circ}$ C, 20 s at 60  $^{\circ}$ C, and 30 s at 72  $^{\circ}$ C. Samples were run in triplicate and their relative expression was determined by normalizing expression of each target to  $\beta$ -actin. The amplification was monitored on a Roter-Gene realtime PCR apparatus (Roter-Gene, Australia). Primers used in these experiments were shown in Table 2.

#### Statistical analysis

Data analysis was performed using SPSS11.0. All data were expressed as mean  $\pm$  SD. Comparison of the differences between each group was performed by  $\chi^2$  test. A *P* value of < 0.05 was considered statistically significant.

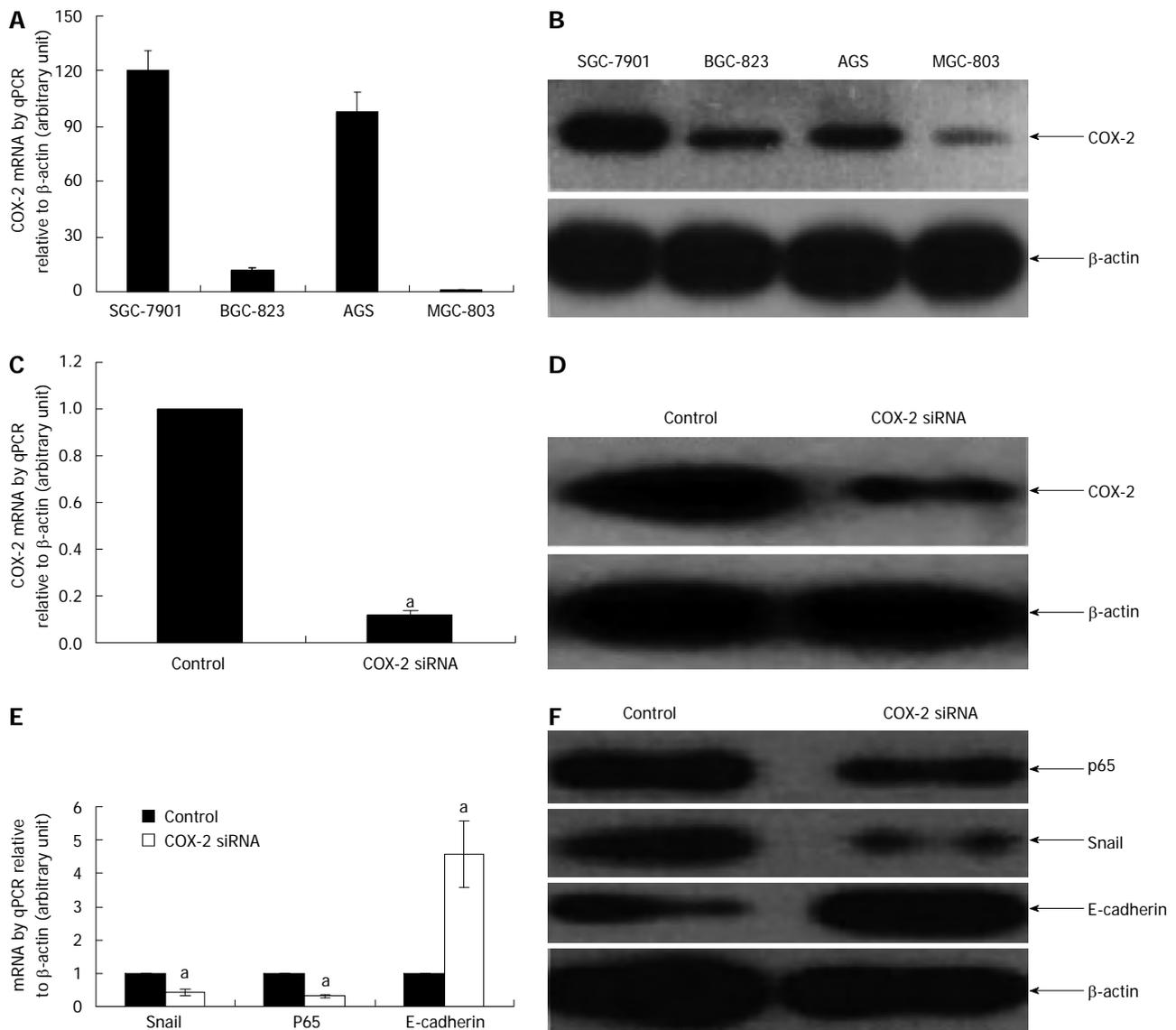
## RESULTS

#### COX-2 baseline expression in human gastric cancer cell lines

We first examined the basal level of COX-2 expression in several human gastric cancer cell lines using qPCR and Western blot. The cell lines tested include SGC-7901 (moderately differentiated), BGC-823 (poorly differentiated), MGC803 (undifferentiated), and AGS (well differentiated). Highest expression level of COX-2 was found in SGC-7901 cells, both at mRNA and protein levels (Figure 1A and B) (*P* < 0.05). Thus, the subsequent experiments were performed in SGC-7901 unless otherwise stated.

#### Effect of COX-2 silencing on NF- $\kappa$ B, Snail, and E-cadherin in SGC7901 cells

In order to test the effect of siRNA-mediated down-regulation of COX-2 on NF- $\kappa$ B, Snail and E-cadherin, SGC-7901 cells were incubated with COX-2 specific siRNA (COX-2-siRNA) and the target gene expression was examined by qPCR and Western blot. As shown in Figure 1, knockdown of COX-2 (Figure 1C and D) led to a 3-fold and 2.3-fold decrease but a 4.6-fold increase in the mRNA expression of NF- $\kappa$ B, Snail, and E-cadherin, respectively (Figure 1E) (*P* < 0.05, compared to their respective controls). These changes were confirmed at the protein level: COX-2-siRNA led to 2.3-fold and 2.8-fold



**Figure 1** Effect of cyclooxygenase-2 knockdown on the expression of nuclear factor- $\kappa$ B, Snail, and E-cadherin in gastric cancer cells. Among the four human gastric cancer cell lines, SGC-7901 has the highest expression level of cyclooxygenase-2 (COX-2) at mRNA (A) and protein level (B). Thus, this cell line was used to study the regulatory effect of COX-2 on nuclear factor- $\kappa$ B (NF- $\kappa$ B), Snail, and E-cadherin. Successful knockdown of COX-2 was confirmed at mRNA (C) and protein (D) levels. Down-regulation of COX-2 led to a reduction of NF- $\kappa$ B subunit p65 and Snail but an increased E-Cadherin, both at the mRNA (E) and protein (F) levels. mRNA expression was examined by quantitative polymerase chain reaction (qPCR) and expressed as a relative arbitrary unit against that of  $\beta$ -actin. Protein expression was examined by Western blot ( $^*P < 0.05$  vs their respective controls).

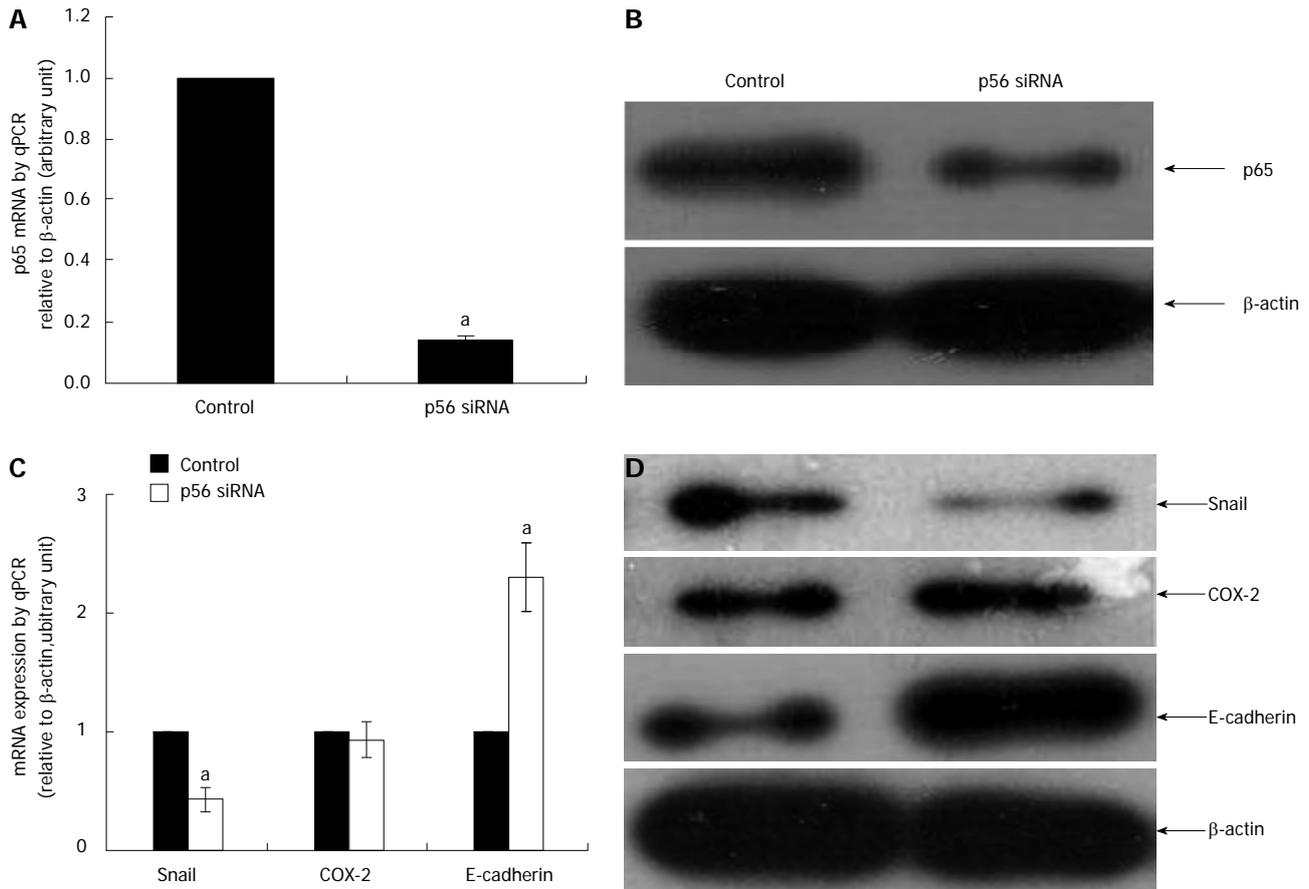
decrease but a 2.5-fold increase in the expression of NF- $\kappa$ B, Snail, and E-cadherin, respectively (Figure 1F) ( $P < 0.05$ , compared to their respective controls).

**Effect of NF- $\kappa$ B silencing on COX-2, Snail and E-cadherin in SGC7901 cells**

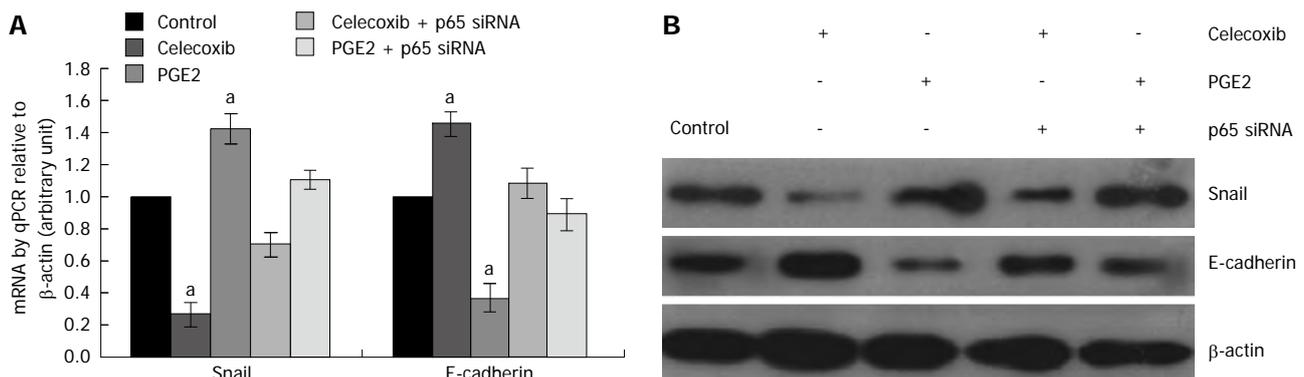
As noted above, siRNA mediated down-regulation of COX-2 led to a reduced expression of NF- $\kappa$ B and Snail in SGC-7901 cells. In order to confirm if COX-2 functions upstream of NF- $\kappa$ B, we examined if NF- $\kappa$ B was able to modulate COX-2 expression in SGC-7901 cells. As shown in Figure 2, knockdown of NF- $\kappa$ B subunit p65 using its specific siRNA (p65-siRNA) (Figure 2A and B) did not affect the expression of COX-2 at both

mRNA and protein levels (Figure 2C and D) ( $P < 0.05$ , compared to their respective controls).

We then proposed that NF- $\kappa$ B could regulate the expression of E-cadherin *via* the transcription factor Snail. Therefore, the effect of NF- $\kappa$ B silencing on Snail and E-cadherin were further examined in SGC-7901 cells. As shown in Figure 2, knockdown of NF- $\kappa$ B (Figure 2A and B) was associated with a reduced expression of Snail at both mRNA and protein levels (Figure 2C and D) ( $P < 0.05$ , compared to their respective controls). On the other hand, blockade of NF- $\kappa$ B with p65-siRNA rendered an increase in the expression of E-cadherin at both mRNA and protein levels (Figure 2C and D) ( $P < 0.05$ , compared to their respective controls).



**Figure 2** Effect of nuclear factor- $\kappa$ B knockdown on the expression of cyclooxygenase-2, Snail, and E-cadherin in SGC-7901 cells. Cells transfected with specific siRNA against nuclear factor- $\kappa$ B (NF- $\kappa$ B) subunit p65 (p65-siRNA) showed a marked down-regulation of p65 at mRNA (A) and protein (B) levels. p65-siRNA led to a reduction of Snail but an increased E-cadherin, both at the mRNA (C) and protein (D) levels. However, p65-siRNA mediated down-regulation of NF- $\kappa$ B did not significantly alter the expression of COX-2, both at the mRNA (C) and protein (D) levels. mRNA expression was examined by polymerase chain reaction (qPCR) and expressed as a relative arbitrary unit against that of  $\beta$ -actin. Protein expression was examined by Western blot. ( $^eP < 0.05$  vs their respective controls).



**Figure 3** Down-regulation of nuclear factor- $\kappa$ B by p65-siRNA reversed the regulatory effect of celecoxib and prostaglandin E2 on Snail and E-cadherin in SGC-7901 cells. Treatment of SGC-7901 cells with celecoxib led to a reduced expression of Snail but an increased expression of E-cadherin both at mRNA (A) and protein (B) levels. In contrast, treatment of SGC-7901 cells with prostaglandin E2 (PGE2) led to an increased Snail and a decreased E-cadherin at mRNA (A) and protein (B) levels. However, when the cells were pre-treated with p65-siRNA, the observed effects of Celecoxib and PGE2 were reversed (A, B). mRNA expression was examined by polymerase chain reaction (qPCR) and expressed as a relative arbitrary unit against that of  $\beta$ -actin. Protein expression was examined by Western blot ( $^eP < 0.05$  vs their respective controls).

**NF- $\kappa$ B inhibition interrupted the effects of celecoxib and PGE2 on E-cadherin and Snail in SGC-7901 cells**

To further determine the regulatory role of NF- $\kappa$ B on E-cadherin, we used celecoxib, a potent COX-2 inhibitor,

and PGE2, a principal COX-2 substrate with reported role in promoting cell migration and invasion in tumors, to treat SGC-7901 cells in the presence or absence of p65-siRNA.

As shown in Figure 3, treatment of SGC-7901 cells with celecoxib led to a reduced expression of Snail but an increased expression of E-cadherin both at mRNA (Figure 3A) and protein (Figure 3B) levels. In contrast, treatment of SGC-7901 cells with PGE2 led to an increased Snail and a decreased E-cadherin at mRNA (Figure 3A) and protein levels (Figure 3B). However, when the cells were pre-treated with p65-siRNA, the observed effects of celecoxib and PGE2 were reversed (Figure 3A and B) ( $P < 0.05$ , compared to their respective controls).

## DISCUSSION

Abnormal down-regulation of E-cadherin is an important event involved in epithelial-mesenchymal transition, a critical process in the malignant transformation of epithelial cancers including gastric cancer<sup>[10,18]</sup>. Previous studies, including our own, have demonstrated that COX-2 has a modulatory effect on expression of E-cadherin in gastric cancer and other malignancies<sup>[12]</sup>. The regulatory role of COX-2 on the expression of E-cadherin is also reflected by the observed chemopreventive effect of the selective COX-2 inhibitor celecoxib which was shown to inhibit the migration and metastasis of tumor cells by up-regulating E-cadherin<sup>[19]</sup>, and further supported by the fact that PGE-2 was able to promote the tumor invasion through down-regulating E-cadherin<sup>[20]</sup>. However, the mechanisms responsible for the regulatory effect of COX-2 on E-cadherin have not been well defined.

E-cadherin is usually lost in gastric cancer tissues and this appeared to be mediated by COX-2<sup>[21]</sup>. In our previous study, we found that inhibition of COX-2 activity by celecoxib was not only associated with a reduced expression of Snail, but also a marked reduction in NF- $\kappa$ B subunit p65<sup>[12]</sup>. In the current study, we explored the same regulatory effect based on RNAi technique. The results showed that COX-2 mediated down-regulation of E-cadherin appeared to be dependent on a functional NF- $\kappa$ B pathway, as blockade of COX-2 activity, either by COX-2-siRNA or celecoxib, restored the expression of E-cadherin. This was associated with a marked down-regulation of NF- $\kappa$ B and Snail expression. These findings are in agreement with previous reports that NF- $\kappa$ B up-regulates Snail and consequently represses E-cadherin in tumor cells<sup>[21,22]</sup>. Snail has been firmly established as a repressor of E-cadherin and it down-regulates E-cadherin transcription through an interaction with proximal E-boxes of the E-cadherin promoter<sup>[23]</sup>. In our current study, we further revealed that blockade of NF- $\kappa$ B by p65-siRNA did not alter the expression of COX-2 in SGC-7901 cells. However, the effect of celecoxib and PGE2 on Snail and E-cadherin was reversed by p65-siRNA, suggesting that a functional COX-2 was necessary for regulating NF- $\kappa$ B and Snail signaling in gastric cancer.

The regulatory role of NF- $\kappa$ B on COX-2 has been reported in other human tumors<sup>[24]</sup>. For example, NF- $\kappa$ B was found to enhance the expression of COX-2 and promote cells proliferation in human colorectal carci-

noma cells<sup>[25]</sup>. In our study, NF- $\kappa$ B p65 was not found to regulate the expression of COX-2. This inconsistency may reflect a cell type specific difference. Additionally, the regulatory role of NF- $\kappa$ B on COX-2 in gastric cancer through other subunits could not be excluded. More studies are needed to unveil the possible mechanisms of how COX-2 and NF- $\kappa$ B interact during gastric cancer formation.

In conclusion, this study has provided further evidence that COX-2 functions upstream of NF- $\kappa$ B in the regulation of Snail and E-cadherin in gastric cancer cells. Blockade of COX-2 activity or inhibition of PGE2 production may offer some benefit in the chemoprevention and treatment of gastric cancer.

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## COMMENTS

### Background

Cyclooxygenase-2 (COX-2) plays an important role in transcriptional regulation of E-cadherin in gastric cancer and other malignancies. celecoxib, a selective inhibitor of COX-2, inhibits migration and metastasis of tumor cells by up-regulation of E-cadherin. On the contrary, prostaglandin E2 (PGE2) promotes invasion of tumor cells through down regulating the expression of E-cadherin. This study aims to explore how COX-2/PGE2 regulates E-cadherin expression and further to determine whether COX-2/PGE2 reduces the expression of E-cadherin via nuclear factor- $\kappa$ B (NF- $\kappa$ B)/ Snail signal pathway in gastric cancer cells.

### Research frontiers

Although the correlation between COX-2 and E-cadherin is always inverse in tumor cells, the mechanism of how COX-2 regulates E-cadherin is not clear yet.

### Innovations and breakthroughs

The authors firstly found COX-2 baseline expression was significantly higher in SGC-7901 cells in comparison to that in BGC-823, MGC-803 and AGS cells. celecoxib or COX-2 specific RNAi both down-regulated NF- $\kappa$ B and Snail expression, and up-regulated E-cadherin expression, in contrast to PGE2, in SGC-7901 cells. Next, they found that NF- $\kappa$ B specific RNAi did not influence the expression of COX-2 in SGC-7901 cells. Therefore, they can conclude preliminarily that NF- $\kappa$ B and Snail are the downstream molecules in COX-2 modulated E-cadherin signaling pathway in SGC-7901 cells.

### Applications

This study has provided further evidence that COX-2 functions upstream of NF- $\kappa$ B in the regulation of Snail and E-cadherin in gastric cancer cells. Blockade of COX-2 activity or inhibition of PGE2 production may offer some benefit in the chemoprevention and treatment of gastric cancer.

### Terminology

Epithelial-mesenchymal transition (EMT): The epithelial-mesenchymal transition is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells. EMT is essential for numerous developmental processes including mesoderm formation and neural tube formation. EMT has also been shown to occur in wound healing, in organ fibrosis and in the initiation of metastasis for cancer progression. COX-2: COX-2 is an inducible isozyme of cyclooxygenase and catalyzes PGE2 formation in response to various inflammatory stimuli or growth factors; E-cadherin: E-cadherin is an important cell adhesion molecule and is usually low, mutated, or even lost in gastric cancer tissues. COX-2 and E-cadherin appear to exhibit totally different expression patterns in tumor cells.

### Peer review

This is a very interesting paper on the molecular biology of COX-2 and E-cadherin via the NF- $\kappa$ B and Snail pathways. The methodology and reasoning is

sound along with the results and logical discussion at the end.

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## Laparoscopic vs open distal pancreatectomy for solid pseudopapillary tumor of the pancreas

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### Abstract

**AIM:** To compare short- and long-term outcomes of laparoscopic vs open distal pancreatectomy for solid pseudopapillary tumor (SPT) of the pancreas.

**METHODS:** This retrospective study included 28 patients who underwent distal pancreatectomy for SPT of the pancreas between 1998 and 2012. The patients were divided into two groups based on the surgical approach: the laparoscopic surgery group and the open surgery group. The patients' demographic data, operative results, pathological reports, hospital courses, morbidity and mortality, and follow-up data were compared between the two groups.

**RESULTS:** Fifteen patients with SPT of the pancreas underwent laparoscopic distal pancreatectomy (LDP), and 13 underwent open distal pancreatectomy (ODP). Baseline characteristics were similar between the two groups except for a female predominance in the LDP

group (100.0% vs 69.2%,  $P = 0.035$ ). Mortality, morbidity (33.3% vs 38.5%,  $P = 1.000$ ), pancreatic fistula rates (26.7% vs 30.8%,  $P = 0.728$ ), and reoperation rates (0.0% vs 7.7%,  $P = 0.464$ ) were similar in the two groups. There were no significant differences in the operating time (171 min vs 178 min,  $P = 0.755$ ) between the two groups. The intraoperative blood loss (149 mL vs 580 mL,  $P = 0.002$ ), transfusion requirement (6.7% vs 46.2%,  $P = 0.029$ ), first flatus time (1.9 d vs 3.5 d,  $P = 0.000$ ), diet start time (2.3 d vs 4.9 d,  $P = 0.000$ ), and postoperative hospital stay (8.1 d vs 12.8 d,  $P = 0.029$ ) were significantly less in the LDP group than in the ODP group. All patients had negative surgical margins at final pathology. There were no significant differences in number of lymph nodes harvested (4.6 vs 6.4,  $P = 0.549$ ) between the two groups. The median follow-up was 33 (3-100) mo for the LDP group and 45 (17-127) mo for the ODP group. All patients were alive with one recurrence.

**CONCLUSION:** LDP for SPT has short-term benefits compared with ODP. Long-term outcomes of LDP are similar to those of ODP.

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**Key words:** Solid pseudopapillary tumor; Pancreatic tumor; Laparoscopic surgery; Distal pancreatectomy

**Core tip:** Solid pseudopapillary tumor (SPT) of the pancreas is a rare neoplasm. Laparoscopic distal pancreatectomy (LDP) and open distal pancreatectomy (ODP) for SPT have not previously been compared. We compared the short-term and long-term outcomes among patients undergoing either LDP or ODP for SPT. Our results showed that LDP for SPT had the advantages of minimally invasive surgery, less intraoperative blood loss, and rapid recovery. The mortality, morbidity, oncological outcome, and long-term outcome of LDP were similar to those of open surgery.

Zhang RC, Yan JF, Xu XW, Chen K, Ajoodhea H, Mou YP. Laparoscopic vs open distal pancreatectomy for solid pseudopapillary tumor of the pancreas. *World J Gastroenterol* 2013; 19(37): 6272-6277 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6272.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6272>

## INTRODUCTION

Solid pseudopapillary tumor (SPT) of the pancreas is a rare neoplasm, accounting for 0.17%-2.7% of all pancreatic tumors, and affecting predominantly young women<sup>[1]</sup>. Frantz<sup>[2]</sup> first described the tumor in 1959 as a papillary tumor of the pancreas. The tumor has been named as a papillary epithelial neoplasm, solid and cystic tumor, solid and papillary tumor, papillary cystic tumor, and solid and papillary epithelial neoplasm depending on its histological features including cystic, solid, and pseudopapillary structures<sup>[1,3]</sup>. In 1996, the World Health Organization renamed this tumor as SPT<sup>[4]</sup>. SPT is of unclear histopathogenesis, and low-grade malignancy, malignant degeneration and lymph node metastasis rarely occur<sup>[1,5]</sup>. Surgical resection of this tumor could result in long-term survival<sup>[1]</sup>.

Laparoscopic resection of the pancreas, including enucleation, pancreaticoduodenectomy, and distal and central pancreatectomy, has been recently described; some of the patients could have benefited from these procedures<sup>[6-10]</sup>. Until April 2013, about 86 cases of laparoscopic/robot-assisted resection for SPT have been reported in the English-language literature. Most of these are case reports and small series. However, there are few reports comparing short-term and long-term outcomes among patients who underwent laparoscopic distal pancreatectomy (LDP) vs open distal pancreatectomy (ODP) for SPT of the pancreas.

The goal of the present study was to compare short-term and long-term outcomes in patients undergoing either LDP or ODP for SPT of the pancreas.

## MATERIALS AND METHODS

### *Patient sample and data collection*

Between May 1998 and December 2012, 55 patients underwent pancreatectomy for SPT of the pancreas at Sir Run Run Shaw Hospital, Hangzhou, China. We retrieved 29 patients who underwent distal pancreatectomy. One patient with liver metastasis and colon cancer was excluded from the study, and 28 patients were included in this study. The medical records of all patients were retrospectively reviewed, including demographics, clinical presentation, operative results, hospital course, morbidity and mortality, pathological findings, and long-term follow-up data. The Institutional Review Board of Sir Run Run Shaw Hospital of Zhejiang University approved this study protocol.

### *Surgical procedure*

All operations were performed by four experienced surgeons using our institution's standardized technique. Laparoscopic pancreatic surgery was adopted in 2003 at our institution, therefore, all of the patients who underwent surgery from 1998 to 2003 were included in the open surgery group. After 2003, the surgeons could decide whether to perform laparoscopic or open surgery with the informed consent of the patients.

### *Operative technique used for distal pancreatectomy*

The operative procedure for LDP has been described previously<sup>[11,12]</sup>. Briefly, the patient was placed in supine position with the head slightly elevated. The surgeon and the second assistant who held the laparoscope stood on the right side of the patient and the first assistant stood on the left. One initial 10-mm trocar was placed for laparoscopy below the umbilicus. A 30-degree telescope was inserted to examine the peritoneal cavity to rule out metastatic disease. After general examination, the other four trocars (one 12 mm, three 5 mm) were inserted into the left upper flank, left flank, right upper flank, and right flank quadrants; and the five trocars were arranged in a V shape. Under pneumoperitoneum, the gastrosplenic ligament was divided for entrance to the lesser sac using a harmonic scalpel (Harmonic Ace; Ethicon Endo-Surgery, Cincinnati, OH, United States). The mobilization of the pancreas began at the superior border until the proximal splenic artery was visualized. The pancreas was mobilized at the inferior border to visualize the superior mesenteric and splenic veins. After creating a tunnel behind the neck of the pancreas, the pancreas was transected with an endoscopic linear stapler (Endocutter 60 stapler, white or blue cartridge; Ethicon Endo-Surgery, Cincinnati, OH, United States). For spleen-preserving procedures, the distal pancreas was freely dissected from the splenic vessels by ligation of the small branches connected to the pancreas using small titanium vascular clips or a harmonic scalpel. In the case of DP with splenectomy, the splenic artery and splenic vein were divided. The spleen was resected with the pancreas.

ODP was performed in the same manner as LDP through an upper midline incision. However, a variety of techniques, including suturing and/or stapling, were used to control the pancreas stump, according to the preference of the individual surgeon.

### *Postoperative management*

Diet was started after the first flatus had been passed. Patients were discharged if they considered themselves sufficiently recovered; tolerated food without any significant discomfort; and had no major complications. Postoperative pancreatic fistula was defined as any measurable volume of drainage fluid (amylase > 3 times the upper limit of normal serum value) on or after postoperative day 3<sup>[13]</sup>. Three different grades of postoperative pancreatic fistula (A-C) were defined according to the clinical impact on the patient's hospital course<sup>[13]</sup>. Postoperative mortality

**Table 1** Baseline characteristics of patients undergoing laparoscopic distal pancreatectomy or open distal pancreatectomy for pancreatic solid pseudopapillary tumor *n* (%)

Characteristics	LDP ( <i>n</i> = 15)	ODP ( <i>n</i> = 13)	<i>P</i> value
Age (yr)	35.4 ± 13.0	35.2 ± 16.6	0.965
Sex			0.035
Male	0 (0.0)	4 (30.8)	
Female	15 (100.0)	9 (69.2)	
BMI (kg/m <sup>2</sup> )	20.8 ± 2.3	22.4 ± 6.1	0.392
Symptoms			0.255
No	10 (66.7)	5 (38.5)	
Yes	5 (33.3)	8 (61.5)	
Comorbidity	4 (26.7)	4 (30.8)	1.000
ASA score			1.000
1	9 (60.0)	8 (61.5)	
2	6 (40.0)	5 (38.5)	
Tumor size (cm)	5.1 ± 1.6	7.7 ± 4.1	0.050
Spleen preservation			0.639
No	13 (86.7)	10 (76.9)	
Yes	2 (13.3)	3 (23.1)	
Combined resection			1.000
Gallbladder	0 (0.0)	1 (7.7)	
Gastric stromal tumor	1 (6.7)	0 (0.0)	

Data are expressed as *n* (%) or mean ± SD or unless otherwise specified. BMI: Body mass index; ASA: American Society of Anesthesiologists; LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy.

was defined as death occurring within 30 d after surgery.

### Patient follow-up

Patients were followed up as outpatients by telephone. We included data up to the last follow-up in March 2013. Recurrence or distant metastasis was diagnosed pathologically by surgical resection, biopsy, or cytology and/or radiological examination. The fasting blood glucose level (normal ≤ 110 mg/dL) was used to evaluate pancreatic endocrine function. The clinical evaluation was used to assess the pancreatic exocrine function. Patients with diarrhea, weight loss, and fatty stools were considered to have pancreatic exocrine insufficiency.

### Statistical analysis

Continuous clinicopathological data were expressed as median (range) or mean ± SD as appropriate. Categorical variables were reported as number and percentage. Continuous clinicopathological data were analyzed with Student's *t* test (or Mann-Whitney *U* test). Categorical variables were analyzed with the  $\chi^2$  test (or Fisher's exact test). All statistical analyses were performed using SPSS version 16.0. *P* < 0.05 was considered statistically significant.

## RESULTS

### Baseline characteristics

Table 1 summarizes the baseline characteristics of the LDP and ODP groups. Fifteen patients underwent LDP and 13 ODP. The two groups were balanced in terms of their baseline characteristics: age, body mass index (BMI), symptoms, comorbidity, American Society of Anesthesiologists (ASA) score, tumor size, spleen preservation

**Table 2** Surgical outcomes of laparoscopic distal pancreatectomy and open distal pancreatectomy for pancreatic solid pseudopapillary tumor *n* (%)

Outcomes	LDP ( <i>n</i> = 15)	ODP ( <i>n</i> = 13)	<i>P</i> value
Operating time (min)	171 ± 54	178 ± 75	0.755
EBL (mL)	149 ± 127	580 ± 400	0.002
Transfused patients	1 (6.7)	6 (46.2)	0.029
First flatus time (d)	1.9 ± 0.5	3.5 ± 0.9	0.000
Diet start time (d)	2.3 ± 0.7	4.9 ± 2.1	0.000
Postoperative hospital stay (d)	8.1 ± 1.7	12.8 ± 6.8	0.029
Morbidity	5 (33.3)	5 (38.5)	1.000
Pancreatic fistula	4 (26.7)	4 (30.8)	0.972
Grade A	2 (13.3)	2 (15.4)	
Grade B	0 (0.0)	0 (0.0)	
Grade C	2 (13.3)	2 (15.4)	
Intra-abdominal abscess	1 (6.7)	0 (0.0)	1.000
Pleural effusion	0 (0.0)	1 (7.7)	0.464
Reoperation	0 (0.0)	1 (7.7)	0.464
Percutaneous drainage	2 (13.3)	2 (15.4)	1.000
Mortality	0 (0.0)	0 (0.0)	-

Data are expressed as *n* (%) or mean ± SD or unless otherwise specified. EBL: Estimated blood loss; LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy.

rate, and combined resection rate, except for a significant female predominance in the LDP group: 100% women (*n* = 15) compared with 69.2% (*n* = 9) in the ODP group (*P* = 0.035).

### Surgical outcomes in the LDP and ODP groups

Table 2 summarizes the operative outcomes and hospital courses of the LDP and ODP groups. There were no significant differences in the operating time (171 min *vs* 178 min, *P* = 0.755) between the two groups. LDP produced a significantly lower amount of intraoperative blood loss (149 mL *vs* 580 mL, *P* = 0.002), lower transfusion requirement (6.7% *vs* 46.2%, *P* = 0.029), shorter first flatus time (1.9 d *vs* 3.5 d, *P* = 0.000), shorter diet start time (2.3 d *vs* 4.9 d, *P* = 0.000), and shorter postoperative hospital stay (8.1 d *vs* 12.8 d, *P* = 0.029) than ODP.

There were no significant differences in postoperative complication rates (33.3% *vs* 38.5%, *P* = 1.000), pancreatic fistula rates (26.7% *vs* 30.8%, *P* = 0.972), and reoperation rates (0.0% *vs* 7.7%, *P* = 0.464) between the two groups. One patient underwent laparotomy for acute peritonitis after open spleen-preserving DP. We found biliary and pancreatic fistulas from the pancreatic stump. A calculus (diameter 6 mm) was incarcerated in the distal common bile duct, which led to bile regurgitation through the pancreaticobiliary common channel. The procedure consisted of cholecystectomy, common bile duct exploration, T tube drainage, and suture of the pancreatic remnant. The patient was discharged 24 d after the second operation. No perioperative mortality was recorded.

### Pathological characteristics

Table 3 shows the pathological characteristics of the two groups. All patients had negative surgical margins at final

**Table 3** Pathological characteristics of patients undergoing laparoscopic distal pancreatectomy or open distal pancreatectomy for pancreatic solid pseudopapillary tumor *n* (%)

Characteristics	LDP ( <i>n</i> = 15)	ODP ( <i>n</i> = 13)	<i>P</i> value
Harvested lymph nodes	4.6 ± 4.1	6.4 ± 6.2	0.549
Negative surgical margin	15 (100.0)	13 (100.0)	-
Invasion of peripancreatic tissue	4 (26.7)	2 (15.4)	0.655
Perineural invasion	1 (6.7)	1 (7.7)	1.000
Liver metastasis	0 (0.0)	0 (0.0)	-
Lymphatic metastasis	0 (0.0)	0 (0.0)	-
Invasion of adjacent organs	0 (0.0)	0 (0.0)	-
Angioinvasion	0 (0.0)	0 (0.0)	-

Data are expressed as *n* (%) or mean ± SD or unless otherwise specified. LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy.

pathology. An average number of 5.3 lymph nodes were resected without metastases. There was no significant difference in the number of harvested lymph nodes (4.6 *vs* 6.4, *P* = 0.549) between the two groups. In seven (25%) patients, the pathological findings were consistent with malignant features of SPT<sup>[14]</sup>. The malignant features included local invasion of peripancreatic tissue (*n* = 6), perineural invasion (*n* = 2), no liver metastasis, invasion of adjacent organs and angioinvasion. There were no significant differences in the pathological characteristics between the two groups.

### Long-term outcomes

The median follow-up was 33 (3-100) mo for the LDP group and 45 (17-127) mo for the ODP group. All patients were alive with one recurrence. A 57-year-old female patient underwent ODP, and the pathology report revealed SPT with peripancreatic tissue invasion and perineural invasion. Six years after surgery, she developed peritoneal recurrence, which was treated by open tumorectomy and traditional Chinese medicine. At a follow-up of 15 mo after the second operation, no tumor recurrence was found. After surgery, six patients developed pancreatic exocrine or endocrine insufficiency; two received pancreatic enzyme therapy; and one developed diabetes and received insulin therapy. There were three cases of hyperglycemia with diet control.

## DISCUSSION

SPT is an uncommon pancreatic neoplasm with nonspecific symptoms or completely asymptomatic<sup>[1]</sup>. A review of 718 patients with SPT showed that the most common localization of the tumor was the distal pancreas [tail (247 patients, 35.9%), body (102 patients, 14.8%), and body and tail (71 patients, 10.3%)]<sup>[1]</sup>. This was also demonstrated in our series (29 patients, 52.7%). Therefore, DP with/without splenectomy is the most common surgical procedure for SPT. Complete resection of SPT offers benefits in almost all patients, and extensive lymphatic dissection is not indicated<sup>[1,15]</sup>. With the feasibility

and safety of LDP being proven<sup>[16,17]</sup>, it seems that LDP is thought to be more appropriate for SPT of the distal pancreas.

The first surgical resection of a pancreatic SPT was performed in 1970 and laparoscopic SPT resection in 2003<sup>[18,19]</sup>. The first series of laparoscopic SPT resection (10 cases) was published by Cavallini *et al*<sup>[20]</sup> in 2011. They regarded that LDP was a safe and feasible procedure for patients with SPT. However, no comparative analysis with open surgery was done. Kang *et al*<sup>[21]</sup> found smaller tumor size, earlier oral intake, and shorter hospital stay, without increasing morbidity in the laparoscopic (8 cases)/robot-assisted (3 cases) surgery group (*P* < 0.05) compared with open surgery group. To the best of our knowledge, the present series is the largest comparison of LDP and ODP for SPT. Our results indicated that LDP for SPT was associated with less operative blood loss and transfusion requirement, earlier first flatus and diet start, and shorter hospital stay compared to ODP, without increasing surgery-related risks (Table 2). The pathological examination showed that LDP for SPT provided similar oncological outcomes (harvested lymph nodes and margin status) as compared with ODP (Table 3). Long-term outcomes of laparoscopic surgery were comparable to those of open surgery. We believe that LDP for SPT could produce better short-term outcomes than ODP, without affecting oncological and long-term outcomes.

Our data and literature<sup>[1]</sup> showed that patients with SPT are expected to have a long-term survival after resection. At a median follow-up of 39 mo, 6 patients developed pancreatic exocrine or endocrine insufficiency. Thus, quality of life should be considered when choosing surgical procedure. Function-preserving laparoscopic pancreatectomy, including laparoscopic central pancreatectomy (LCP), spleen-preserving (SP)-LDP is thought to be an ideal procedure for this tumor. Some experts have reported the surgical technique of LCP with operative outcomes in small case series<sup>[9,10]</sup>. Three patients with SPT underwent LCP in our center. Nevertheless, the number of patients was too small to draw any conclusion. With the advances in instrumentation and accumulating experience, LCP would be an alternative procedure for SPT in the neck or proximal body of the pancreas.

As compared with SP-LDP, LDP with splenectomy tends to impair quality of life, with frequent higher-grade complications and prolonged hospital stays<sup>[22]</sup>. Butturini *et al*<sup>[23]</sup> compared the results of patients who underwent SP-LDP with or without splenic vessel conservation, and showed that postoperative morbidity did not differ between the two groups. The rate of perigastric varices was 60.0% after splenic vessel resection and 21.7% after splenic vessel conservation (*P* = 0.123)<sup>[23]</sup>. No gastrointestinal bleeding occurred at a median follow-up of 69 (37-139) mo<sup>[23]</sup>. In our series, only two patients underwent SP-LDP with splenic vessel conservation and 13 patients underwent LDP with splenectomy. For the small number of cases, there was no comparability between SP-LDP with splenic vessel conservation group and LDP with

splenectomy group. Considering the low malignancy of SPT and high rate of perigastric varices after splenic vessel resection, it is best to try to preserve the spleen with splenic vessels.

Recently, Fais *et al*<sup>[24]</sup> reported three patients with recurrences within 3 years after resection for SPT (laparoscopic biopsy with resection in one case, and laparoscopic biopsy and open resection in two cases). They considered that recurrence after laparoscopic biopsy may be due to diffusion of tumor cells caused by gas insufflation<sup>[24]</sup>. In our series, 15 patients underwent LDP without biopsy or broken specimen. At a median follow-up of 33 mo, all patients were alive without recurrence. In our opinion, laparoscopic biopsy should not be performed in patients with SPT. During laparoscopic surgery, we should make sure that the integrity of the specimen is not broken.

The limitations of this study were its retrospective design and low number of patients. These problems can be overcome only by a large, prospective randomized trial, which would be difficult to accomplish owing to the infrequent diagnosis of patients with SPT of the distal pancreas. We believe that this study could provide useful evidence in clinical practice.

In conclusion, LDP for SPT is feasible and safe, and has short-term benefits compared with ODP. Long-term outcomes are similar for LDP and ODP.

## COMMENTS

### Background

Solid pseudopapillary tumor (SPT) of the pancreas is a rare neoplasm. Some patients have benefited from laparoscopic pancreatectomy. Laparoscopic distal pancreatectomy (LDP) has not previously been compared with open distal pancreatectomy (ODP) for SPT.

### Research frontiers

Recently, several case reports and small series have shown less intraoperative blood loss and rapid recovery after LDP for SPT. However, the short-term and long-term outcomes of LDP compared with ODP for SPT required further assessment.

### Innovations and breakthroughs

In the present study, the authors compared the short-term and long-term outcomes of LDP and ODP for SPT, and showed that LDP was suitable and minimally invasive for treating SPT and could achieve similar oncological outcomes (harvested lymph nodes and margin status) and long-term outcomes as ODP.

### Applications

This study showed that LDP for SPT had the advantages of minimally invasive surgery, less intraoperative blood loss, and rapid recovery. The mortality, morbidity, oncologic outcomes and long-term results of LDP were similar to those of ODP. These findings are helpful in decision-making for the treatment of SPT of the distal pancreas.

### Peer review

The topic is interesting, and despite the rarity of SPT, the study includes a large series of patients.

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## De novo combined lamivudine and adefovir dipivoxil therapy vs entecavir monotherapy for hepatitis B virus-related decompensated cirrhosis

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### Abstract

**AIM:** To compare efficacy of combined lamivudine (LAM) and adefovir dipivoxil (ADV) therapy with that of entecavir (ETV) monotherapy for hepatitis B virus (HBV)-related decompensated liver cirrhosis.

**METHODS:** A total of 120 naive patients with HBV-related decompensated cirrhosis participated in this study. Sixty patients were treated with combined LAM and ADV therapy (LAM + ADV group), while the other 60 were treated with ETV monotherapy (ETV group) for two years. Tests for liver and kidney function, alpha-

fetoprotein, HBV serum markers, HBV DNA load, prothrombin time (PT), and ultrasonography or computed tomography scan of the liver were performed every 1 to 3 mo. Repeated measure ANOVA and the  $\chi^2$  test were performed to compare the efficacy, side effects, and the cumulative survival rates at 48 and 96 wk.

**RESULTS:** Forty-five patients in each group were observed for 96 wk. No significant differences in HBV DNA negative rates and alanine aminotransferase (ALT) normalization rates at weeks 48 ( $\chi^2 = 2.12$  and 2.88) and 96 ( $\chi^2 = 3.21$  and 3.24) between the two groups were observed. Hepatitis B e antigen seroconversion rate in the LAM + ADV group at week 96 was significantly higher in the ETV group (43.5% vs 36.4%,  $\chi^2 = 4.09$ ,  $P < 0.05$ ). Viral breakthrough occurred in 2 cases (4.4%) by week 48 and in 3 cases (6.7%) by week 96 in the LAM + ADV group, and no viral mutation was detected. In the ETV group, viral breakthrough occurred in 1 case (2.2%) at the end of week 96. An increase in albumin ( $F = 18.9$  and 17.3), decrease in total bilirubin and in ALT ( $F = 16.5$ , 17.1 and 23.7, 24.8), reduced PT ( $F = 22.7$  and 24.5), and improved Child-Turcotte-Pugh and the model for end-stage liver disease scores ( $F = 18.5$ , 17.8, and 24.2, 23.8) were observed in both groups. The cumulative rates of mortality and liver transplantation were 16.7% (10/60) and 18.3% (11/60) in the LAM + ADV and ETV groups, respectively.

**CONCLUSION:** Both LAM + ADV combination therapy and ETV monotherapy can effectively inhibit HBV replication, improve liver function, and decrease mortality.

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**Key words:** Chronic hepatitis B; Decompensated liver cirrhosis; Lamivudine; Adefovir dipivoxil; Combination therapy; Entecavir

**Core tip:** This study compared the *de novo* efficacy of combined lamivudine (LAM) and adefovir dipivoxil (ADV) therapy with that of entecavir (ETV) monotherapy for patients with hepatitis B virus (HBV)-related decompensated liver cirrhosis. Both LAM + ADV combination therapy and ETV monotherapy can effectively inhibit HBV replication, improve liver function, and decrease mortality. The data obtained in this study demonstrate the efficacy and the safety of these treatment regimens for 96 wk in patients with HBV-related decompensated liver cirrhosis.

Lian JS, Zeng LY, Chen JY, Jia HY, Zhang YM, Xiang DR, Yu L, Hu JH, Lu YF, Zheng L, Li LJ, Yang YD. *De novo* combined lamivudine and adefovir dipivoxil therapy vs entecavir monotherapy for hepatitis B virus-related decompensated cirrhosis. *World J Gastroenterol* 2013; 19(37): 6278-6283 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6278.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6278>

## INTRODUCTION

Cirrhosis is the end stage of chronic liver damage and is characterized by fibrosis resulting in the distortion and destruction of normal liver architecture. Functional liver tissue is destroyed and replaced by regenerating nodules that do not fully restore lost liver function. Cirrhosis may be due to various causes, including hepatitis B virus (HBV) infection, hepatitis C (HCV) and alcohol consumption<sup>[1,2]</sup>. Chronic infection with HBV accounts for 30% of hepatic cirrhosis globally<sup>[3]</sup>. In China, about 93 million people are carriers of HBV, with 20 million people chronically infected. Within a five-year period, 10% to 20% of patients with chronic hepatitis B develop cirrhosis<sup>[4]</sup>. The five-year survival rates of patients with compensated cirrhosis and of those with decompensated cirrhosis (determined by the presence of ascites, hepatoencephalopathy, and/or history of variceal bleeding) were 84% and 14%, respectively<sup>[5]</sup>. Cirrhosis precedes most cases of hepatocellular carcinoma (HCC), with 70% to 90% of HCC developing from liver cirrhosis or inflammation<sup>[6]</sup>. Antiviral agents are assumed to reduce decompensated cirrhosis and HCC development<sup>[7]</sup>, however, agents such as lamivudine (LAM) and telbivudin show high drug resistance. The latest chronic hepatitis B prevention and treatment guidelines suggest the selection of a higher genetic barrier to resistant antivirals, such as entecavir (ETV) and tenofovir, for patients with HBV-related liver cirrhosis<sup>[8,9]</sup>. However, based on the paradigm that drug combination therapy is more effective than monotherapy for the treatment of human immunodeficiency virus and HCV, the same approach may be appropriate for chronic hepatitis B. This study was designed to compare the two-year efficacy of *de novo* combination therapy of LAM and adefovir dipivoxil (ADV) with that of ETV monotherapy in patients with decompensated liver cirrhosis.

## MATERIALS AND METHODS

### Study patients

From January 2008 to March 2009, 120 patients diagnosed with HBV-related decompensated liver cirrhosis at the First Affiliated Hospital of the Zhejiang University School of Medicine (Hangzhou, China) were recruited into this study. The diagnosis was based on medical history, the results of physical examination, biochemical, endoscopic and ultrasound findings, and radiological signs of cirrhosis. All patients were 18 to 65 years old, with  $\geq 10^3$  copies/mL HBV DNA, 7 to 12 (inclusive) Child-Turcotte-Pugh (CTP) score,  $\geq 50$  mL calculated serum creatinine clearance,  $\geq 75$  g/L hemoglobin,  $\geq 2.5 \times 10^9$ /L total white blood cells,  $\leq 20$  ng/mL  $\alpha$ -fetoprotein, and no evidence of HCC. None of the patients had been treated with antiviral drugs, including interferon- $\alpha$  or nucleos(t)ides. Patients with hepatitis delta virus, hepatitis C virus, or had human immunodeficiency virus (HIV) co-infection were excluded. Patients with HCC, autoimmune hepatitis, alcoholic liver cirrhosis, hepatorenal syndrome, grade 3 or 4 hepatic encephalopathy, spontaneous bacterial peritonitis, and severe heart, renal, and brain diseases were also excluded. All patients who participated in this study provided informed consent and were aware of the procedures to be conducted. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University.

### Study design

The study was designed as a prospective case-control study. The patients were randomly assigned to the ETV monotherapy (60 patients) group and the *de novo* LAM and ADV combination therapy (60 patients) group. Baseline data of the two groups were compared to ensure comparability. Patients in the combination therapy group were prescribed 100 mg LAM and 10 mg ADV per day, while the monotherapy group received 0.5 mg ETV per day.

### Follow-up studies

Serum hepatitis B viral markers, including hepatitis B surface antigen (HBsAg), antibody to HBsAg, hepatitis B e antigen (HBeAg), antibody to HBeAg and antibody to hepatitis B core antigen, were detected by commercially available enzyme immunoassays (Abbott Laboratories; Chicago, IL, United States). Serum HBV DNA was measured by polymerase chain reaction with a linear range between  $1 \times 10^3$  and  $5 \times 10^8$  copies/mL (Shanghai ZJ Bio-Tech Co., Ltd., China).

Follow-up observations in the two groups were performed at the start and during weeks 4, 12, 24, 36, 48, 60, 72, 84, and 96. Follow-up clinical assessments included physical examination, HBeAg and antibodies to the e antigen, quantitative HBV DNA, serum biochemistry, alpha-fetoprotein, renal function, prothrombin time (PT), and ultrasonography or computed tomography scan. The lower limit of detection of DNA used in this study was  $1.0 \times 10^3$  copies/mL (Shanghai ZJ Bio-Tec Co., Ltd, Chi-

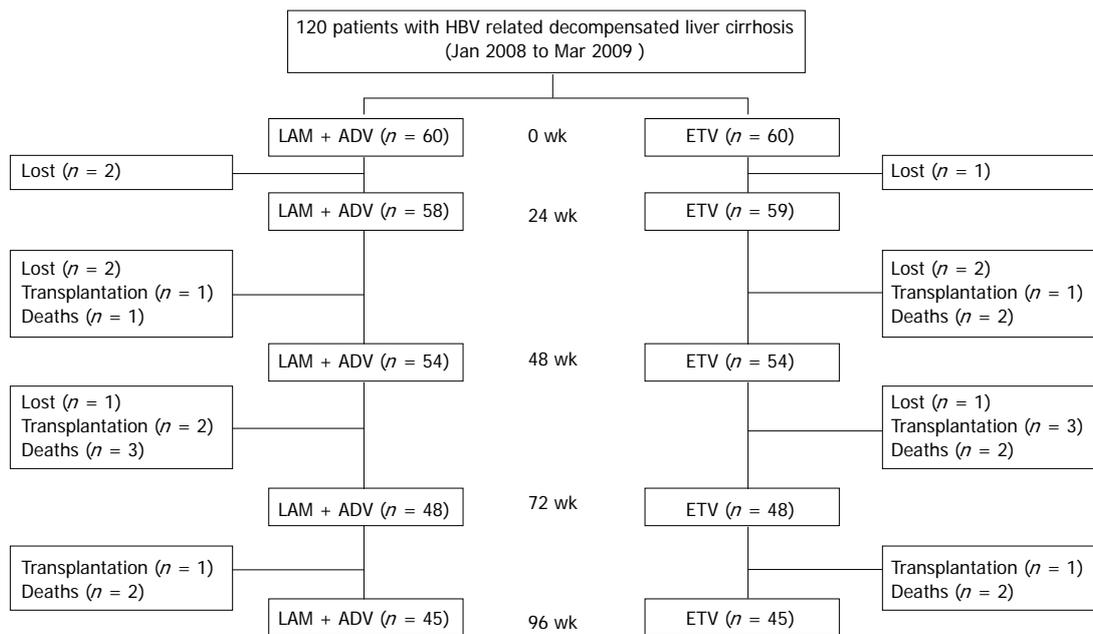


Figure 1 Flow chart of patient conditions following treatment with *de novo* lamivudine and adefovir dipivoxil combination therapy and entecavir monotherapy for 96 wk. LAM: Lamivudine; ADV: Adefovir dipivoxil; ETV: Entecavir.

na). The condition of the patients after 96 wk is shown in Figure 1.

### Statistical analysis

SPSS 16.0 software was used for data analysis. Measurements were presented as mean  $\pm$  SD and comparisons were conducted following analysis of the results using the Student's *t* test. Proportions were presented as percentage (%). Rate comparisons were performed using the  $\chi^2$  test. A *P* value < 0.05 was considered significant.

## RESULTS

### Baseline characteristics

In the two years of follow-up observations, of the 60 patients who received LAM and ADV combination therapy, 5 cases were lost, 4 cases underwent liver transplantation, 6 cases died, and 45 cases survived until the end of the observation period. The 45 remaining cases comprised 16 females and 29 males. The mean patient age was  $53.1 \pm 8.8$  years. Of the 60 patients who received ETV monotherapy, 4 cases were lost, 5 cases underwent liver transplantation, 6 cases died, and 45 cases survived until the end of the observation period. The 45 remaining cases comprised 17 females and 28 males. The mean patient age was  $53.2 \pm 7.4$  years. The baseline characteristics of the patients were similar, and no statistically significant differences were observed (Table 1).

### Virological, serological and biochemical response

Of the 45 patients in the LAM and ADV combination group, 51.1% (23/45) and 86.7% (39/45) achieved undetectable HBV DNA by weeks 48 and 96, respectively. Of the 45 patients in the ETV group, 60% (27/45) and

88.9% (40/45) achieved undetectable HBV DNA by weeks 48 and 96, respectively. No statistical differences were observed between the two groups by weeks 48 and 96 (*P* > 0.05).

In the LAM and ADV combination therapy group, 71.1% (32/45) and 88.9% (40/45) of patients achieved ALT normalization by week 48 and 96, respectively. In the ETV treatment group, 68.9% (31/45) and 91.1% (41/45) achieved ALT normalization by week 48 and 96, respectively. No statistical difference was observed between the two groups at week 48 and 96 (*P* > 0.05).

Of the 45 patients who received the LAM and ADV combination treatment, 30.4% (7/23) and 43.5% (10/23) achieved HBeAg seroconversion by weeks 48 and 96, respectively. Similarly, 27.3% (6/22) and 36.4% (8/22) of the patients who received ETV monotherapy achieved HBeAg seroconversion by weeks 48 and 96, respectively. No statistical difference was observed between the two groups at week 48, while the HBeAg seroconversion rate in the LAM and ADV combination group at week 96 was significantly higher than that in the ETV monotherapy group (43.5% vs 36.4%,  $\chi^2 = 4.09$ , *P* < 0.05).

Of the respondents, 2 and 3 patients in the LAM and ADV combination group and 1 and 2 patients in the ETV monotherapy group developed virological breakthrough by weeks 48 and 96, respectively. No genetic mutations were detected in either patient group. The obtained differences were not statistically different (*P* > 0.05).

### Changes in liver function in patients with decompensated cirrhosis

After 96 wk of treatment, the albumin level in patients in the LAM and ADV combination group increased sig-

**Table 1** Baseline characteristics of patients with HBV-related decompensated cirrhosis *n* (%)

Variables	LAM + ADV ( <i>n</i> = 45)	ETV ( <i>n</i> = 45)	<i>t</i>	$\chi^2$	<i>P</i> value
Age (yr)	53.1 ± 8.8	53.2 ± 7.4	0.23		> 0.05
Male/female	29/16	28/17		3.10	> 0.05
HBV DNA (log <sub>10</sub> copy/mL)	6.56 ± 1.13	6.61 ± 1.15	0.33		> 0.05
ALT (U/L)	98.1 ± 21.6	99.8 ± 17.2	0.23		> 0.05
TBil (μmol/L)	51.6 ± 8.9	49.2 ± 6.8	0.31		> 0.05
Alb (g/L)	29.2 ± 0.7	29.5 ± 1.2	0.24		> 0.05
PT (s)	16.3 ± 2.3	16.5 ± 1.9	0.21		> 0.05
CTP score	8.4 ± 1.7	8.6 ± 2.1	0.16		> 0.05
MELD score	13.7 ± 3.5	12.9 ± 6.7	0.25		> 0.05
HBeAg positive rate	23 (51.1)	22 (48.9)		2.13	> 0.05
Ascites	22 (48.9)	21 (46.7)		2.46	> 0.05
HE	6 (13.3)	7 (15.5)		3.13	> 0.05
UGB	9 (22.2)	8 (17.8)		3.35	> 0.05

ALT: Alanine aminotransferase; TBil: Total bilirubin; Alb: Albumin; PT: Prothrombin time; CTP: Child-Turcotte-Pugh; MELD: Model for end-stage liver disease; HE: Hepatic encephalopathy; UGB: Upper gastrointestinal bleeding; HBeAg: Hepatitis B e antigen.

nificantly compared with the baseline level ( $F = 18.9$ ,  $P < 0.05$ ), whereas ALT and TBil decreased significantly compared with the baseline levels ( $F = 16.5$  and  $23.7$ , respectively,  $P < 0.05$ ). In addition, PT was significantly shortened ( $F = 22.7$ ,  $P < 0.05$ ), and both CTP and MELD scores decreased significantly compared with the baseline scores ( $F = 18.5$  and  $24.2$ , respectively,  $P < 0.05$ ). A decrease of more than 2 points in the CTP score in 31 (68.9%) cases was observed and is shown in Table 2.

After 96 wk, patients who received ETV treatment exhibited a significant increase in albumin level compared with the baseline level ( $F = 17.3$ ,  $P < 0.05$ ). In contrast, ALT and TBil decreased significantly compared with baseline levels ( $F = 17.1$  and  $24.8$ ,  $P < 0.05$ ). PT was significantly shortened ( $F = 24.5$ ,  $P < 0.05$ ), and CTP and MELD scores decreased significantly compared with the baseline levels ( $F = 17.8$  and  $23.8$ ,  $P < 0.05$ ). A decrease in the CTP score by more than 2 points was evident in 30 (66.7%) cases.

The LAM and ADV combination group and the ETV monotherapy group showed no significant differences in albumin level or in ALT, TBil, PT, CTP, and MELD scores by weeks 48 and 96 (Table 2).

### Adverse events

All patients in this study responded well to both LAM and ADV combination therapy and ETV monotherapy. Creatinine levels in four cases in the LAM and ADV combination therapy group and in one case in the ETV monotherapy group were more than twice the baseline values, but were still lower than upper limit of normal. No patient developed lactic acidosis in either group.

In the combination group, the cumulative mortality (including liver transplantation) was 16.7% (10/60) during the follow-up period, and included 2 cases of upper gastrointestinal bleeding, 2 cases of hepatic encephalopathy, 1 case of secondary bacterial infection, and 1 case of

hepatorenal syndrome. Four patients had undergone liver transplantation in this group. In the ETV monotherapy group, the cumulative mortality (including liver transplantation) was 18.3% (11/60), and included 3 cases of upper gastrointestinal bleeding, 2 cases of secondary bacterial infection, and 2 cases of hepatorenal syndrome. Three patients had undergone liver transplantation. These findings are illustrated in Figure 2.

## DISCUSSION

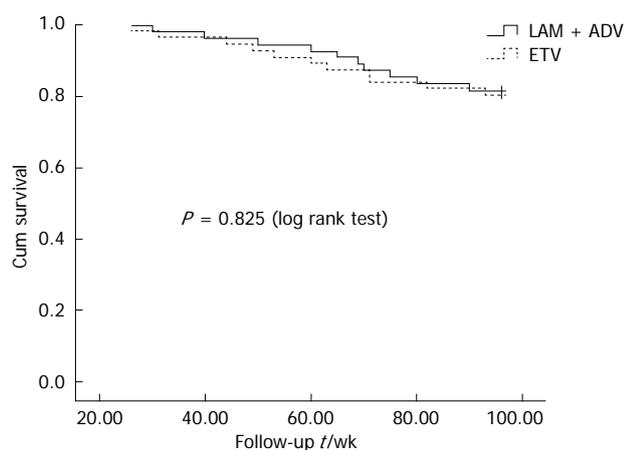
Increasing evidence shows that suppression of HBV replication results in the reduction of hepatic necroinflammation and consequently, improvement of liver function in patients with HBV-related decompensated liver cirrhosis. Antiviral therapy associated with improved outcomes in patients with HBV-related decompensated cirrhosis, including postponement or prevention of liver transplantation, reducing the incidence of HCC<sup>[10-12]</sup>.

LAM was the first oral agent approved for the treatment of chronic hepatitis B (CHB) and currently has a well-established safety and efficacy profile. Liaw *et al*<sup>[13]</sup> reported that continuous treatment with LAM delays clinical progression of CHB infection in patients by significantly reducing the incidence of hepatic decompensation and HCC. ADV benefits pre- and post-transplant patients with LAM-resistant CHB, including decompensated cirrhotics, by suppressing HBV DNA and by improving the CTP score<sup>[14,15]</sup>. In China, ADV is relatively cheap and a large number of CHB patients, including cirrhotics, have received LAM and ADV combination therapy. According to the latest guidelines, the patients with liver cirrhosis and those who have received a liver graft for HBV-related cirrhosis should be considered for *de novo* combination therapy because of the risk of clinical deterioration if they develop drug resistance<sup>[16]</sup>. But the data to support a role of combination therapy in these patients were limited. On the other hand, ETV demonstrates very low rates of resistance in nucleoside-naïve patients and is recommended for patients with HBV-related decompensated cirrhosis<sup>[8,17]</sup>. Therefore, a comparison of the efficacy and safety between LAM and ADV combination therapy and ETV monotherapy for patients with HBV-related decompensated cirrhosis is urgent. Our study showed that 51.1% and 86.7% of patients in the LAM and ADV combination group achieved undetectable HBV DNA by weeks 48 and 96, respectively, while 60% and 88.9% of patients in the ETV treatment group achieved undetectable HBV DNA by weeks 48 and 96, respectively. In addition, both *de novo* combination of LAM and ADV therapy and ETV monotherapy significantly increased albumin level and decreased TBil, PT, CTP, and MELD scores compared with baseline. More importantly, 68.9% of patients in the combination group and 66.7% of patients in the monotherapy group had a decrease in their CTP score of more than 2 points after 96 wk of treatment. A total of 73.7% of patients in the combination group and 71.1% of patients in the monotherapy group exhibited an increase in the CTP score at the end of 96

**Table 2** Comparison of changes in hepatic function

Characteristics	LAM + ADV combination group			ETV monotherapy group		
	0 wk	48 wk	96 wk	0 wk	48 wk	96 wk
Alb (g/L)	29.2 ± 0.7	32.2 ± 0.5	36.7 ± 0.2 <sup>a</sup>	28.9 ± 1.2	31.9 ± 0.4	36.4 ± 0.6 <sup>a</sup>
TBil (μmol/L)	51.6 ± 8.9	30.8 ± 7.5	19.1 ± 6.2 <sup>a</sup>	47.2 ± 6.8	31.6 ± 6.8	18.2 ± 3.9 <sup>a</sup>
ALT (U/L)	98.1 ± 21.6	56.1 ± 21.3	34.7 ± 12.8 <sup>a</sup>	99.8 ± 17.2	54.2 ± 15.7	32.5 ± 11.5 <sup>a</sup>
PT (s)	16.3 ± 2.3	14.3 ± 1.6	12.6 ± 2.1 <sup>a</sup>	16.5 ± 1.9	13.8 ± 2.0	12.9 ± 3.7 <sup>a</sup>
CTP score	8.4 ± 1.7	6.8 ± 1.9	5.5 ± 3.7 <sup>a</sup>	8.6 ± 2.1	6.7 ± 2.5	5.7 ± 1.3 <sup>a</sup>
MELD score	13.7 ± 3.5	9.8 ± 3.1	7.6 ± 1.8 <sup>a</sup>	12.9 ± 6.7	9.6 ± 4.3	7.9 ± 2.3 <sup>a</sup>

<sup>a</sup>*P* < 0.05 vs baseline. ALT: Alanine aminotransferase; TBil: Total bilirubin; Alb: Albumin; PT: Prothrombin time; CTP: Child-Turcotte-Pugh; MELD: Model for end-stage liver disease; LAM: Lamivudine; ADV: Adefovir dipivoxil; ETV: Entecavir.



**Figure 2** Kaplan-Meier analysis of cumulative survival rate in patients with hepatitis B virus-related decompensated cirrhosis treated with lamivudine and adefovir dipivoxil combination therapy and entecavir monotherapy for 96 wk. LAM: Lamivudine; ADV: Adefovir dipivoxil; ETV: Entecavir.

wk. No genetic mutations in either treatment group were detected. In this study, no statistically significant difference was observed between the LAM and ADV combination therapy group and the ETV monotherapy group in terms of the serological conversion rate by 48 wk, while the HBeAg seroconversion rate in the LAM and ADV combination group at week 96 was significantly higher than that in the ETV group. This finding is similar to the results of previous studies<sup>[18]</sup>.

HBV-related decompensated cirrhosis requires a longer duration of antiviral therapy and consideration of the effect and safety of these drugs are essential. LAM has been shown to be safe. ADV, in contrast, is mainly excreted by the kidney and has an impact on renal function during long-term antiviral therapy<sup>[19]</sup>. Our study confirms that ADV treatment of decompensated cirrhosis is safe and effective. However, in the subsequent stages of treatment, doctors should closely monitor kidney function and adjust the treatment plan as soon as renal function is found to be abnormal.

The best treatment method for late-stage HBV-related decompensated liver cirrhosis is liver transplantation. However, transplantation is very expensive and there is a worldwide donor shortage. Liver transplantation is considered in the treatment of decompensated cirrhosis only

when the CTP score for grade C or the MELD score is more than 20 points. Upon detection of HBV DNA, patients with decompensated cirrhosis should be immediately treated with antiviral therapy to improve liver function and to reduce the need for liver transplantation.

Lange *et al*<sup>[20]</sup> reported on 16 patients with liver cirrhosis and chronic hepatitis B who were treated with ETV. Five of these patients developed lactic acidosis (all with MELD scores > 20) during ETV treatment. Lactic acidosis was lethal for one patient, while for other patients, the symptoms were resolved after termination/interruption of ETV treatment. In the present study, no cases of lactic acidosis were observed during the follow-up period of 96 wk.

In conclusion, both *de novo* LAM and ADV combination therapy and ETV monotherapy are effective in patients with HBV-related decompensated cirrhosis, with no differences in the level of HBV DNA suppression, liver function improvement, resistance rate, and on confirmed changes in renal parameters and in cumulative survival rate. The data obtained in this study demonstrate the efficacy and the safety of these treatment regimens for 96 wk in patients with HBV-related decompensated liver cirrhosis, as well as their evident therapeutic benefits in both groups.

## COMMENTS

### Background

The mortality rate of hepatitis B virus (HBV)-related decompensated cirrhosis is very high. Recommended treatment options are monotherapy with high genetic barrier nucleos(t)ide analogues or combination therapy with no cross resistance nucleos(t)ide analogues. There has been no report regarding the entecavir monotherapy or *de novo* lamivudine and adefovir dipivoxil combination therapy in these patients.

### Research frontiers

*De novo* combination therapy with lamivudine and adefovir dipivoxil is better than lamivudine monotherapy in patients with HBV-related decompensated liver cirrhosis. But there is no head to head research to compare the entecavir, a high genetic barrier nucleoside analogue monotherapy with *de novo* lamivudine and adefovir dipivoxil combination therapy for those patients. In this study, the authors demonstrated that both entecavir monotherapy and *de novo* lamivudine and adefovir dipivoxil combination therapy were effective for patients with HBV-related decompensated liver cirrhosis.

### Innovations and breakthroughs

Many clinical studies showed that the combined therapy is effective for patients with human immunodeficiency virus and hepatitis C virus infection. And entecavir is effective for patients with HBV-related decompensated liver cirrhosis.

This is the first head to head study to report that both *de novo* lamivudine and adefovir dipivoxil combination therapy and entecavir monotherapy are effective for patients with HBV-related decompensated liver cirrhosis.

### Applications

By understanding that both *de novo* lamivudine and adefovir dipivoxil combination therapy and entecavir monotherapy are effective for patients with HBV-related decompensated liver cirrhosis, this study may represent a future strategy for therapeutic intervention in patients with HBV-related decompensated liver cirrhosis.

### Terminology

*De novo* combination therapy means combination with two or more drugs from the beginning of the treatment. Monotherapy means use one drug from the beginning of the treatment. The diagnosis of decompensated liver cirrhosis was based on clinical, laboratory, previous histological, ultrasonographic and radiological signs of cirrhosis with Child-Turcotte-Pugh (CTP) score. The CTP score is a system to assess the disease stage for decompensated cirrhotic patients.

### Peer review

This is a good clinical study in which the authors compared the effects of *de novo* lamivudine and adefovir dipivoxil combination therapy with entecavir monotherapy for HBV-related decompensated liver cirrhosis patients. The authors concluded that both *de novo* lamivudine and adefovir dipivoxil combination therapy and entecavir monotherapy are effective for patients with HBV-related decompensated liver cirrhosis.

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## Endoscopic ultrasound elastography for differentiating between pancreatic adenocarcinoma and inflammatory masses: A meta-analysis

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### Abstract

**AIM:** To evaluate the accuracy of endoscopic ultrasound (EUS) elastography for differentiating between pancreatic ductal adenocarcinoma (PDAC) and pancreatic inflammatory masses (PIM).

**METHODS:** Electronic databases (updated to December 2012) and manual bibliographical searches were carried out. A meta-analysis of all diagnostic clinical trials evaluating the accuracy of EUS elastography in differentiating PDAC from PIM was conducted. Heterogeneity was assessed among the studies. The meta-analysis was performed to evaluate the accuracy of EUS elastography in differentiating PDAC from PIM in homogeneous studies.

**RESULTS:** Ten studies involving 781 patients were included in the analysis. Significant heterogeneity in

sensitivity was observed among the studies (Cochran  $Q$  test = 24.16,  $df = 9$ ,  $P = 0.0041$ ,  $I^2 = 62.8\%$ ), while heterogeneity in specificity was not observed (Cochran  $Q$  test = 5.93,  $df = 9$ ,  $P = 0.7473$ ,  $I^2 = 0.0\%$ ). The area under the curve under the Sports Rights Owners Coalition was 0.8227. Evaluation of heterogeneity suggested that the different diagnostic standards used in the included studies were the source of heterogeneity. In studies using the color pattern as the diagnostic standard, the pooled sensitivity, specificity, positive likelihood ratio (LR), negative LR and diagnostic OR were 0.99 (0.97-1.00), 0.76 (0.67-0.83), 3.36 (2.39-4.72), 0.03 (0.01-0.07) and 129.96 (47.02-359.16), respectively. In studies using the hue histogram as the diagnostic standard, the pooled sensitivity, specificity, positive LR, negative LR and diagnostic OR were 0.92 (0.89-0.95), 0.68 (0.57-0.78), 2.84 (2.05-3.93), 0.12 (0.08-0.19) and 24.69 (12.81-47.59), respectively.

**CONCLUSION:** EUS elastography is a valuable method for the differential diagnosis between PDAC and PIM. And a preferable diagnostic standard should be explored and improvements in specificity are required.

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**Key words:** Endoscopic ultrasound; Elastography; Pancreatic adenocarcinoma; Meta-analysis

**Core tip:** Pancreatic inflammatory masses (PIM) are easily confused with pancreatic ductal adenocarcinoma (PDAC). Endoscopic ultrasound (EUS) elastography is a promising noninvasive method for differentiating between PDAC and PIM and may prove to be a valuable supplemental method to EUS-guided fine-needle aspiration.

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inflammatory masses: A meta-analysis. *World J Gastroenterol* 2013; 19(37): 6284-6291 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i37/6284.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6284>

## INTRODUCTION

Pancreatic cancer is a highly lethal disease, and approximately 90% of pancreatic tumors are pancreatic ductal adenocarcinoma (PDAC) which has an extremely poor prognosis<sup>[1,2]</sup>. The 5-year survival rate of PDAC is as low as 0.2%<sup>[3]</sup>. The only potentially curable treatment which is surgical resection, relies on early diagnosis<sup>[4]</sup>. Pancreatic inflammatory masses (PIM) are confused with PDAC<sup>[5]</sup>. The differential diagnosis between PDAC and PIM is currently still difficult due to non-specific symptoms, signs or imaging presentations<sup>[6]</sup>.

Endoscopic ultrasound (EUS) elastography is a recently developed technique for the differential diagnosis of benign and malignant pancreatic masses and measures the mechanical properties of tissues<sup>[7-14]</sup>. The tissue elasticity modulus is represented by a transparent color superimposed on the conventional gray-scale B-mode scans. The nature of the tissue is analyzed either by a qualitative method where blue-predominant represents malignancy or a quantitative method where a value of more than 175 represents malignancy.

Pancreatic masses include PDAC, PIM, neuroendocrine tumors, metastatic tumors, lymphoma, sarcoma, insulinoma and lipoma. Several meta-analyses have evaluated the accuracy of EUS elastography in the diagnosis of pancreatic masses. The overall accuracy of EUS elastography in differentiating between PDAC and PIM has not been assessed. The aim of this study was to perform a meta-analysis of existing studies to assess the accuracy of EUS elastography in differentiating between PDAC and PIM.

## MATERIALS AND METHODS

### Study selection

Studies were selected according to the inclusion and exclusion criteria which were delineated prior to the literature search. The inclusion criteria were: (1) diagnostic clinical trials assessing the accuracy of EUS elastography for differentiating between PDAC and PIM; (2) cytology of EUS-guided fine-needle aspiration (FNA) samples, histopathology of surgical specimens or a follow-up period of at least 6 months as a reference standard; and (3) sufficient data to construct a  $2 \times 2$  table for true-positive, false-positive, false-negative and true-negative findings.

Studies were excluded if they met the following criteria: (1) studies without complete data available for constructing a  $2 \times 2$  table for true-positive, false-positive, false-negative and true-negative findings; (2) studies

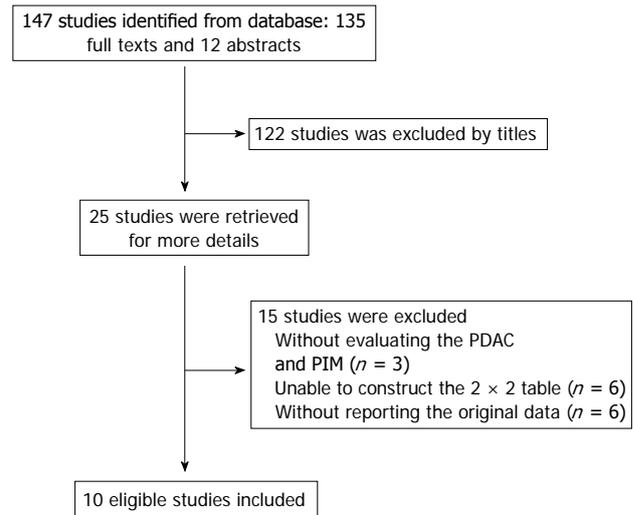


Figure 1 Literature search flow diagram.

updated or duplicated; (3) studies which did not report their own data such as editorials, reviews, corresponding letters; and (4) case reports.

### Literature search

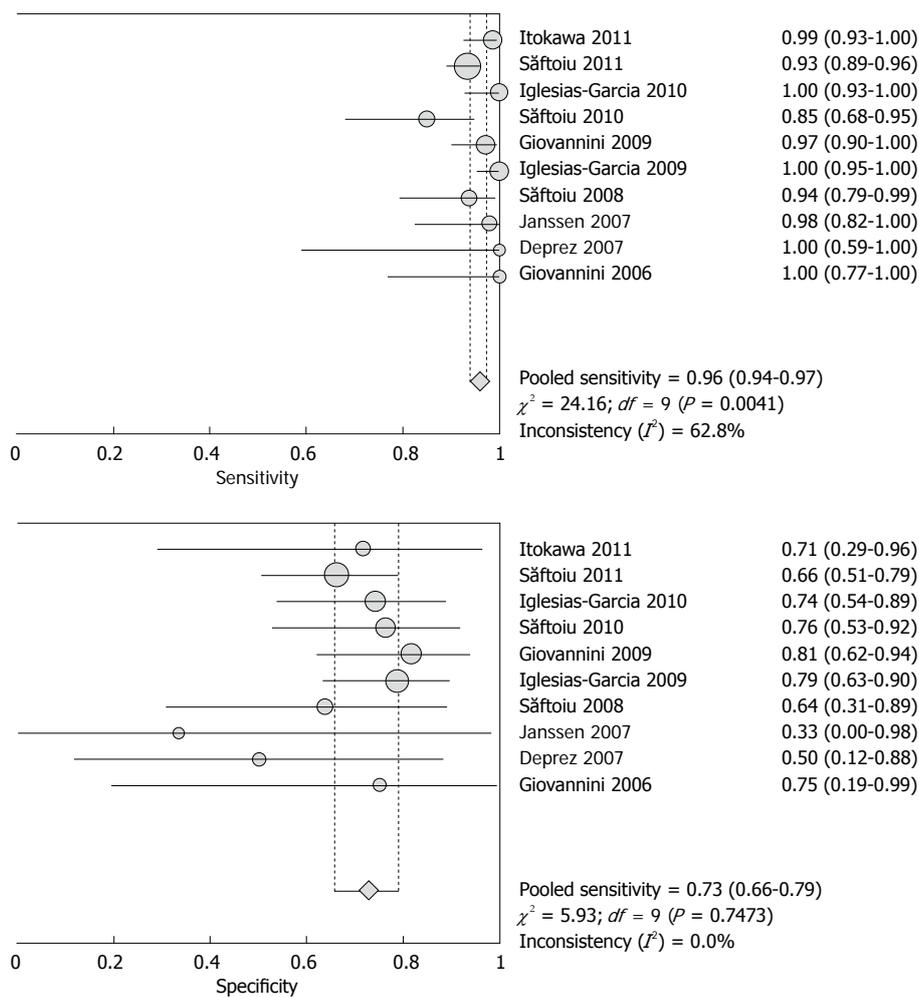
Using the Medline, Embase, Web of Science, and Cochrane Central Trials databases up to Dec. 2012, a systematic literature search was conducted. The search strategy was (“elastogram” or “elastography” or “elastonoendoscopy” or “sonoelastography”) and (“pancreatic” or “pancreas” or “adenocarcinoma” or “inflammatory mass”). To expand the search, we also performed a manual search of abstracts presented at the United European Gastroenterology Week (UEGW) congresses and the American Digestive Disease Week (DDW) from 2000 to 2012. The bibliographies of each peer-reviewed paper were screened for other potentially relevant studies. If missing data were needed, we contacted the appropriate authors by mail.

### Statistical analysis

Data on the differentiation between PDAC and PIM were extracted. The Cochrane  $Q$  test was used to assess heterogeneity with a  $P$  value  $< 0.10$ <sup>[15]</sup>.  $I^2$  was used to describe the percentage variability attributable to heterogeneity rather than sampling errors.  $I^2 > 25\%$  indicated the presence of heterogeneity. The Spearman  $\rho$  between the logit of sensitivity and logit of 1-specificity was calculated to assess the presence of a threshold effect. A strong correlation (Spearman  $\rho < -0.4$ ) suggested the presence of a threshold effect<sup>[16]</sup>. The source of heterogeneity, with the exception of the threshold effect, was explored by meta-regression analysis<sup>[17,18]</sup>. The subgroups were predefined, and included diagnostic standard (color pattern *vs* hue histogram), blind (yes *vs* unclear), sample size ( $\geq 50$  *vs*  $< 50$ ), type of publication (full text *vs* abstract), and design of study (single center *vs* multicenter). A  $P$  value  $< 0.05$  indicated significance. Pooling was only

**Table 1** Baseline characteristics of the studies in the analysis

Ref.	Type of publication	Design of study	Diagnostic standard	Cut-off	No.
Săftoiu <i>et al</i> <sup>[7]</sup>	Full text	Single center	Hue histogram	> 175	54
Iglesias-Garcia <i>et al</i> <sup>[8]</sup>	Full text	Single center	Color pattern	Blue-predominant	76
Janssen <i>et al</i> <sup>[9]</sup>	Full text	Single center	Color pattern	Blue-predominant	25
Deprez <i>et al</i> <sup>[10]</sup>	Abstract	Single center	Color pattern	Blue-predominant	13
Săftoiu <i>et al</i> <sup>[11]</sup>	Full text	Single center	Hue histogram	> 175	43
Iglesias-Garcia <i>et al</i> <sup>[12]</sup>	Full text	Single center	Color pattern	Blue-predominant	119
Giovannini <i>et al</i> <sup>[13]</sup>	Full text	Multicenter	Color pattern	Blue-predominant	96
Giovannini <i>et al</i> <sup>[14]</sup>	Full text	Single center	Color pattern	Blue-predominant	18
Itokawa <i>et al</i> <sup>[24]</sup>	Full text	Single center	Color pattern	Blue-predominant	79
Săftoiu <i>et al</i> <sup>[34]</sup>	Full text	Multicenter	Color pattern	> 175	258



**Figure 2** Forest plot (random-effect model) of the meta-analysis for sensitivity (upper) and specificity (lower) in differentiating between pancreatic ductal adenocarcinoma and pancreatic inflammatory masses.

conducted within the homogeneous groups using the fixed-effect model (Mantel-Haenszel method<sup>[19]</sup>). Pooling the results with corresponding 95%CI included sensitivity, specificity, positive likelihood ratio (LR), negative LR and diagnostic odds ratio (DOR).

In order to analyze the presence of publication bias, funnel plots were constructed using the Harbord<sup>[20]</sup> and Egger indicator and Begg<sup>[21]</sup> and Mazumdar indicator. Asymmetric funnel plots or a *P* value < 0.1 suggested the presence of publication bias. The quality of the se-

lected studies was assessed using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) questionnaire<sup>[22]</sup>. Items were rated as yes, no, or unclear.

The pooled weighted sensitivity, specificity, positive LR, negative LR, DOR, Sports Rights Owners Coalition (SROC) curve and Spearman analysis were performed using Meta-Disc version 1.4 (Unit of Clinical Biostatistics, Ramon y Cajal Hospital, Madrid, Spain)<sup>[23]</sup>. Meta-regression and publication bias analyses were performed using Stata version 10.0 (Stata Corporation, College Sta-

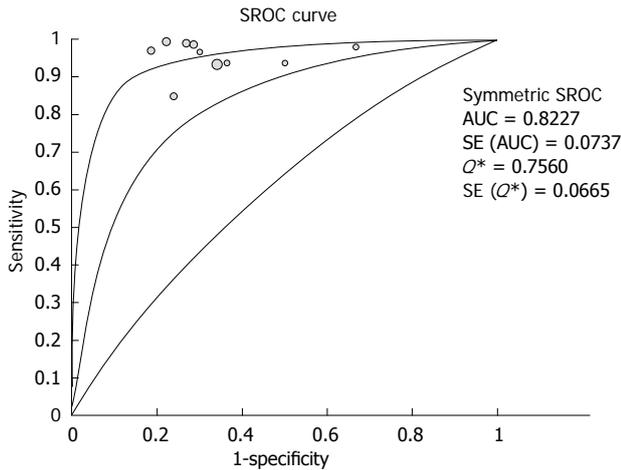


Figure 3 Sports Rights Owners Coalition, with 95%CI, for differentiating between pancreatic ductal adenocarcinoma and pancreatic inflammatory masses.

tion, TX, United States).

## RESULTS

The initial literature search identified a total of 147 studies (Figure 1). Of these 147 studies, 25 potentially relevant studies were retrieved for further evaluation. Ten studies involving 781 patients were finally included in this meta-analysis. The baseline characteristics of the selected studies are listed in Table 1. Nine studies were published as full texts, and 1 as an abstract. Seven studies used the color pattern as the diagnostic standard, while the other three used the hue histogram value.

### Differentiating PDAC and PIM

The pooled sensitivity and specificity (random-effect model) of EUS elastography for differentiating between PDAC and PIM were 96% (95%CI: 94-97) and 73% (95%CI: 66-79), respectively. Significant heterogeneity in sensitivity was observed among the studies (Cochran *Q* test = 24.16, *df* = 9, *P* = 0.0041, *I*<sup>2</sup> = 62.8%), while heterogeneity in specificity was not observed (Cochran *Q* test = 5.93, *df* = 9, *P* = 0.7473, *I*<sup>2</sup> = 0.0%) (Figure 2). The AUC under the SROC was 0.8227 (Figure 3).

By excluding the study reported as an abstract, the pooled sensitivity and specificity (random-effect model) were 96% (95%CI: 94-97) and 73% (95%CI: 66-80), respectively. There was significant heterogeneity in sensitivity among the studies (Cochran *Q* test = 23.56, *df* = 8, *P* = 0.0027, *I*<sup>2</sup> = 66.1%), while heterogeneity in specificity was not observed (Cochran *Q* test = 5.50, *df* = 8, *P* = 0.8090, *I*<sup>2</sup> = 0.0%). The AUC under the SROC was 0.8188.

### Test of heterogeneity

The source of heterogeneity was explored. A Spearman  $\rho$  of -0.29 (*P* = 0.41) between the logit of sensitivity and the logit of 1-specificity did not suggest the presence of

Table 2 Meta-regression analysis for the potential source of heterogeneity

Study characteristics	Z	P value	95%CI
Diagnostic standard (color pattern vs hue histogram)	2.90	0.00	0.68-3.50
Blind (yes vs unclear)	1.36	0.17	-0.87-4.82
Sample size ( $\geq 50$ vs $< 50$ )	0.13	0.90	-1.90-2.17
Type of publication (full text vs abstract)	1.28	0.20	-1.33-6.37
Design of study (single center vs multicenter)	0.04	0.97	-1.35-1.40

Table 3 Subgroup analysis on the basis of the diagnostic standards

Pooled estimate	Color pattern (n = 426) <sup>1</sup>		Hue histogram (n = 355) <sup>2</sup>	
	Pooled result (95%CI)	<i>I</i> <sup>2</sup>	Pooled result (95%CI)	<i>I</i> <sup>2</sup>
Sensitivity	0.99 (0.97-1.00)	0.00%	0.92 (0.89-0.95)	20.10%
Specificity	0.76 (0.67-0.83)	0.00%	0.68 (0.57-0.78)	0.00%
Positive LR	3.36 (2.39-4.72)	17.90%	2.84 (2.05-3.93)	0.00%
Negative LR	0.03 (0.01-0.07)	0.00%	0.12 (0.08-0.19)	0.00%
Diagnostic OR	129.96 (47.02-359.16)	0.00%	24.69 (12.81-47.59)	0.00%

<sup>1</sup>Studies using the color pattern as the diagnostic standard and the total number of patients involved; <sup>2</sup>Studies using the hue histogram as the diagnostic standard and the total number of patients involved. OR: Odds ratio; LR: Likelihood ratio.

a threshold effect. The meta-regression analysis showed that the different diagnostic standards used in the selected studies were the source of heterogeneity (*P* = 0.00). In addition, the characteristics of blinding, sample size, type of publication and design of study were not related to heterogeneity (Table 2).

### Meta-analysis based on diagnostic standards

The evaluation of heterogeneity suggested that the different diagnostic standards used in the included studies were the source of heterogeneity. As a result, the meta-analysis was performed on the studies using the same diagnostic standards. The pooled results showed good homogeneity. Pooling was conducted using the fixed-effect model (Mantel-Haenszel method<sup>[22]</sup>). In studies using the color pattern as the diagnostic standard, the pooled sensitivity, specificity, positive LR, negative LR and DOR were 0.99 (0.97-1.00), 0.76 (0.67-0.83), 3.36 (2.39-4.72), 0.03 (0.01-0.07) and 129.96 (47.02-359.16), respectively. In studies using the hue histogram as the diagnostic standard, the pooled sensitivity, specificity, positive LR, negative LR and DOR were 0.92 (0.89-0.95), 0.68 (0.57-0.78), 2.84 (2.05-3.93), 0.12 (0.08-0.19) and 24.69 (12.81-47.59), respectively (Table 3).

### Quality assessment using the QUADAS questionnaire

The quality of the selected studies according to the QUADAS questionnaire is shown in Figure 4. The overall quality of the studies was good. Eight studies were rated as “yes” in all items. In the study by Janssen *et al*<sup>[9]</sup>

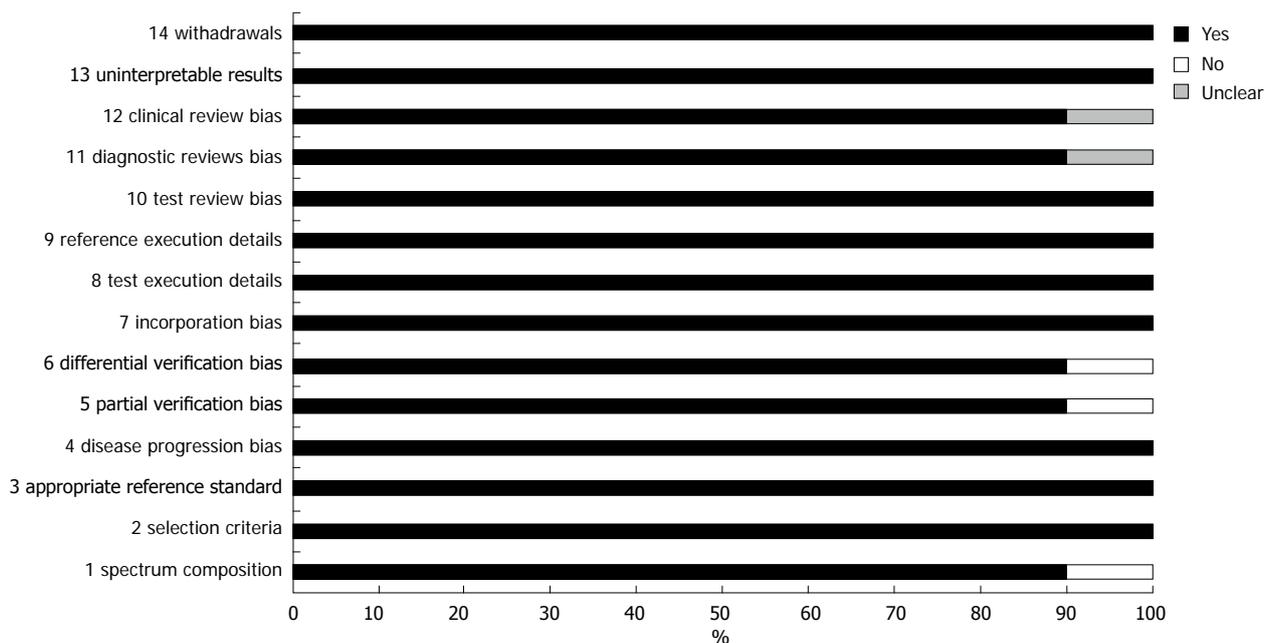


Figure 4 The Quality Assessment of Diagnostic Accuracy Studies scores of the selected studies are summed up per item and presented in a bar chart.

all selected patients were referred for EUS-guided FNA or surgery and one of the selected patients was diagnosed with lipoma by CT densitometry without histological proof. As a result, QUADAS question 1, 5 and 6 were rated as “no”. In addition, Jassen *et al*<sup>[9]</sup> and Itokawa *et al*<sup>[24]</sup> did not mention whether blinding was used in their study. As a result, QUADAS question 11 was rated as “unclear”.

**Publication bias**

The Harbord-Egger indicator for publication bias provided a value of 1.65 (95%CI: -0.43-2.59, *P* = 0.14) and the Begg-Mazumdar indicator gave a Kendall’s tau *b* value of 9 (*P* = 0.47) for the selected studies, which suggested no publication bias (Figure 5).

**DISCUSSION**

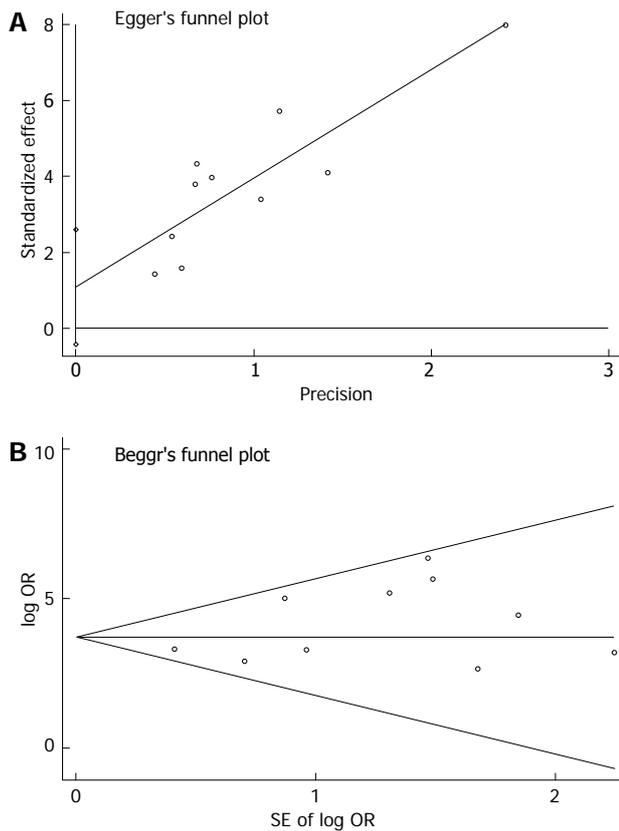
Pancreatic cancer is the fourth leading cause of cancer-related death in the USA, and the second among gastrointestinal tumors<sup>[25]</sup>. Early diagnosis may allow patients to receive the only potentially curable treatment which is surgical resection. PDAC is found in more than 90% of patients with pancreatic cancer and most of the lesions confused with PDAC are benign PIM<sup>[5]</sup>. PDAC is frequently associated with secondary inflammatory changes caused by obstruction of the pancreatic duct. In addition, chronic pancreatitis can markedly increase the risk of PDAC<sup>[26]</sup>. As a result, the differential diagnosis between PDAC and PIM is essential for clinical decision-making.

Despite considerable advances in imaging techniques, the diagnosis of PDAC, particularly in the setting of chronic pancreatitis, remains a challenge. There are no

characteristic findings to differentiate pancreatic masses on transabdominal ultrasound (TAS) and its accuracy is very low<sup>[27]</sup>. Computed tomography (CT) and magnetic resonance imaging (MRI) may be used for staging and detecting metastasis, however, these techniques have limited ability in differentiating between PDAC and PIM<sup>[28,29]</sup>. Endoscopic retrograde cholangiopancreatography (ERCP) has an increased risk of complications, the most important being pancreatitis<sup>[30]</sup>.

EUS, which provides high-resolution images of the pancreas, has become an indispensable tool in the management of pancreatic diseases. However, an important limitation of EUS examination is its low capacity to determine the exact nature of pancreatic masses<sup>[31]</sup>. EUS-FNA allows pathological diagnosis. It is currently considered an accurate and safe method for the diagnosis of pancreatic disease. However, EUS-FNA is an invasive procedure and the sensitivity of EUS-FNA is less than 75% in the presence of coexistent chronic pancreatitis or “pseudotumoral” pancreatitis<sup>[32,33]</sup>.

EUS elastography is a newly developed technique which assesses the mechanical properties of tissues during conventional EUS examination. In this meta-analysis, no significant publication bias was detected using the Harbord-Egger and Begg-Mazumdar indicators. The meta-regression analysis demonstrated that the different diagnostic standards used in the included studies may be the source of heterogeneity. This meta-analysis indicated that EUS elastography could achieve a very high sensitivity and a moderate specificity for differentiating between PDAC and PIM. As EUS elastography showed good sensitivity it may be an appropriate method for monitoring patients with PIM in whom malignancy has been excluded. In addition, it could also be used to fol-



**Figure 5** Funnel plots. A: Funnel plot of the Harbord-Egger indicator for the selected studies; B: Funnel plot of the Begg-Mazumdar indicator for the selected studies.

low patients with PDAC after surgery.

The pooled specificity of EUS elastography may not be satisfactory for differentiating between PDAC and PIM compared with the 100% specificity of EUS-FNA. This may be due to the following reasons: first, diagnostic studies preferred maximal sensitivity in order to reduce the false negative rate when setting up the cutoff value. This reduced the specificity of individual studies. Second, this study focused on the differentiation between PDAC and PIM. The data from normal controls and chronic pancreatitis patients without focal masses, which could easily be excluded from malignancy by EUS elastography, were excluded from this study. This would markedly reduce the number of true negative cases, and thereby decrease specificity.

As an imaging method with moderate specificity, EUS elastography could not replace EUS-FNA which provides a pathological diagnosis. However, it may be a valuable supplemental method to EUS-FNA. EUS elastography and FNA could be performed sequentially during the same EUS procedure. It could be used to guide FNA to reduce the number of false negative cases, especially in patients with coexisting pancreatitis. Moreover, EUS elastography may provide additional information for differentiating between PDAC and PIM when a negative EUS-FNA result is obtained or the patients are

unsuitable for FNA.

The diagnostic standard used for the analysis of mechanical properties was correlated with the accuracy of EUS elastography in the differentiation of pancreatic masses. The qualitative color pattern and quantitative hue histogram value are two currently used diagnostic standards. In general, the quantitative diagnostic standard would be considered better because it is an objective method. Based on unified samples (PDAC and PIM), a subgroup analysis was performed to compare these two standards. The results showed that studies using the color pattern as the diagnostic standard showed preferable pooled estimates than those using the hue histogram. This may be due to the fact that both the overall stiffness and the distribution of stiffness were associated with the nature of the tissue. The color pattern diagnostic standard takes the predominant color and the distribution of the color into consideration simultaneously, while the hue histogram value only gives overall stiffness.

There were some limitations in this meta-analysis. One of the selected studies was published as an abstract, and some details were not available. A small number of studies were included in this study which may have reduced the power of the analysis.

In conclusion, EUS elastography is a valuable method for the differential diagnosis between PDAC and PIM. And a preferable diagnostic standard should be explored and improvements in specificity are required.

## COMMENTS

### Background

Endoscopic ultrasound (EUS) elastography is a recently developed technique for the differential diagnosis of benign and malignant pancreatic masses and measures the mechanical properties of tissues. The overall accuracy of EUS elastography in differentiating between pancreatic ductal adenocarcinoma (PDAC) and pancreatic inflammatory masses (PIM) has not been assessed.

### Research frontiers

Several meta-analyses on the accuracy of EUS elastography in the diagnosis of pancreatic masses have been carried out. The overall accuracy of EUS elastography in differentiating between PDAC and PIM has not been assessed.

### Innovations and breakthroughs

Previous studies have mainly focused on the differential diagnosis of benign and malignant pancreatic masses. This analysis suggested that EUS elastography could achieve a very high sensitivity and a moderate specificity for differentiating PDAC from PIM. Such findings were not presented clearly in previous studies.

### Applications

This analysis suggested that EUS elastography could achieve a very high sensitivity and a moderate specificity for differentiating PDAC from PIM. Due to good sensitivity, EUS elastography may be an appropriate method for monitoring patients with PIM in whom malignancy has been excluded. In addition, it could be used to follow patients with PDAC after surgery.

### Peer review

This is a well-performed meta-analysis of currently available studies on the accuracy of EUS elastography in the differential diagnosis between PDAC and PIM. The authors found that EUS elastography is a promising noninvasive method for differential diagnosis of PDAC and PIM and may prove to be a valuable supplemental method to EUS-guided fine-needle aspiration. This is a good meta-analysis and the authors have included many relevant issues missed by other research groups.

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## Video capsule endoscopy and CT enterography in diagnosing adult hypertrophic pyloric stenosis

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pyloric stenosis; Gastroparesis; Endoscopy; Computed tomography enterography

**Core tip:** Classic descriptors and latest developments and potential role for capsule endoscopy in differential diagnosis of adult hypertrophic pyloric stenosis (HPS). First ever case of 3 years of video capsule retention in a patient. First ever report of diagnosing adult HPS with video capsule and/or with computed tomography-enterography. Physiologic effects of video capsule on symptoms in adult HPS. Differentiating between adult HPS and gastroparesis. Advances in treatment of adult HPS.

Gurvits GE, Tan A, Volkov D. Video capsule endoscopy and CT enterography in diagnosing adult hypertrophic pyloric stenosis. *World J Gastroenterol* 2013; 19(37): 6292-6295 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6292.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6292>

### Abstract

Primary adult hypertrophic pyloric stenosis is a rare but important cause of gastric outlet obstruction that may be misdiagnosed as idiopathic gastroparesis. Clinically, patients present with early satiety, abdominal fullness, nausea, epigastric discomfort and eructation. Permanent gastric retention of a video capsule endoscope is diagnostic in differentiating between the two diseases, in the absence of an organic gastric outlet obstruction. This case presents the longest video capsule retention in the medical literature to date. It is also the first case report of adult hypertrophic pyloric stenosis diagnosed with video capsule endoscopy or a computed tomography scan. Finally, an unusual "plugging" of the gastric outlet with free floating capsule has an augmented effect on disease physiology and on patient's symptoms.

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**Key words:** Video capsule endoscopy; Hypertrophic

### INTRODUCTION

Video capsule endoscopy is an important tool in our ever growing arsenal in detecting bowel related abnormalities. Its wide use has led to the significant improvement of our ability to understand and diagnose a variety of gastrointestinal (GI) diseases. Retention of the capsule is one of the feared complications of the procedure, however, its location can often point to the identifiable pathology in a patient with an obscure GI condition. In this case report, we present a truly unique situation in which a discovery of a retained capsule led to the correct diagnosis of adult hypertrophic pyloric stenosis in a patient previously diagnosed with idiopathic gastroparesis. Interestingly, freely floating video capsule intermittently "plugged" already compromised gastric outlet, resulting in worsening of the patient's symptoms.

## CASE REPORT

A 53-year-old female presented to our office for a second opinion evaluation of a progressive history of early satiety, weight loss, and postprandial abdominal fullness of over 10-year duration, with notable exacerbation of her symptoms over last 3 years. Her symptoms would typically worsen toward the evening. Her past medical history included depression, surgically excised breast cancer, gastroesophageal reflux disease, and eradication of *Helicobacter pylori* in the absence of peptic ulcer disease. Several prior esophagogastroduodenoscopies performed at different clinics were notable for persistent retention of solid food in the stomach despite overnight fasts and nuclear studies showed prolonged gastric emptying. The patient was diagnosed with gastroparesis, however, she failed to respond to trials of metoclopramide, domperidone, or erythromycin. Part of her previous work up also included a video capsule endoscopy in 2008 that commented on delayed gastric transit and an erroneous conclusion of a capsule entering small bowel after 7 h. On our initial physical exam, the patient appeared in no distress. Abdominal evaluation was unremarkable, including absence of a succession splash. Laboratory values were all within normal limits. A scout abdominal roentogram revealed a retained capsule in the gastric antrum, and a barium upper GI series demonstrated pronounced delay in gastric emptying with a narrowed slightly elongated pylorus and a distended gastric antrum (Figure 1A, B). Computed tomography (CT) enterography of the abdomen verified retained video capsule in the distended antrum of the stomach and visualized abnormally dense, eccentric, and significantly narrowed pylorus measuring 15 mm in thickness and 24 mm in length (Figure 1C). Endoscopic findings were remarkable for retained semi-digested food particles despite a two day liquid diet, and confirmed the presence of a 3-year-old video capsule freely floating in the gastric fundus. A notably fixed and narrow (7 mm pre-instrumentation) thickened pyloric channel was successfully traversed with a standard 10 mm endoscope applying moderate pressure (Figure 2). The pyloric mucosa appeared unremarkable with no mass lesion or ulceration. Evaluation of the proximal duodenum was unremarkable. Four quadrant biopsies of the pyloric channel did not reveal malignancy. The video capsule was endoscopically retrieved with a Roth Net<sup>®</sup>. The patient was diagnosed with adult hypertrophic pyloric stenosis that accounted for her symptoms. She refused possible endoscopic interventions with balloon dilation or Botox injection, and declined a surgical pyloroplasty. During follow-up at three and 6 mo, the patient appeared well and reported mild improvement in her symptoms.

## DISCUSSION

Video capsule endoscopy (VCE) has revolutionized noninvasive evaluation of small intestinal mucosa since its approval for clinical use in the United States in 2001. Initially utilized for the assessment of patients with GI

bleeding of obscure origin, its indications have expanded to include evaluation for small bowel tumors, polyposis syndromes, inflammatory bowel disease, and enteropathies, including celiac disease<sup>[1]</sup>. Contraindications for the study include motility disorders, obstruction, known stenotic area in the GI tract, pregnancy<sup>[2]</sup>. Today, capsule endoscopy is used in evaluation of some esophageal and colonic disorders as well.

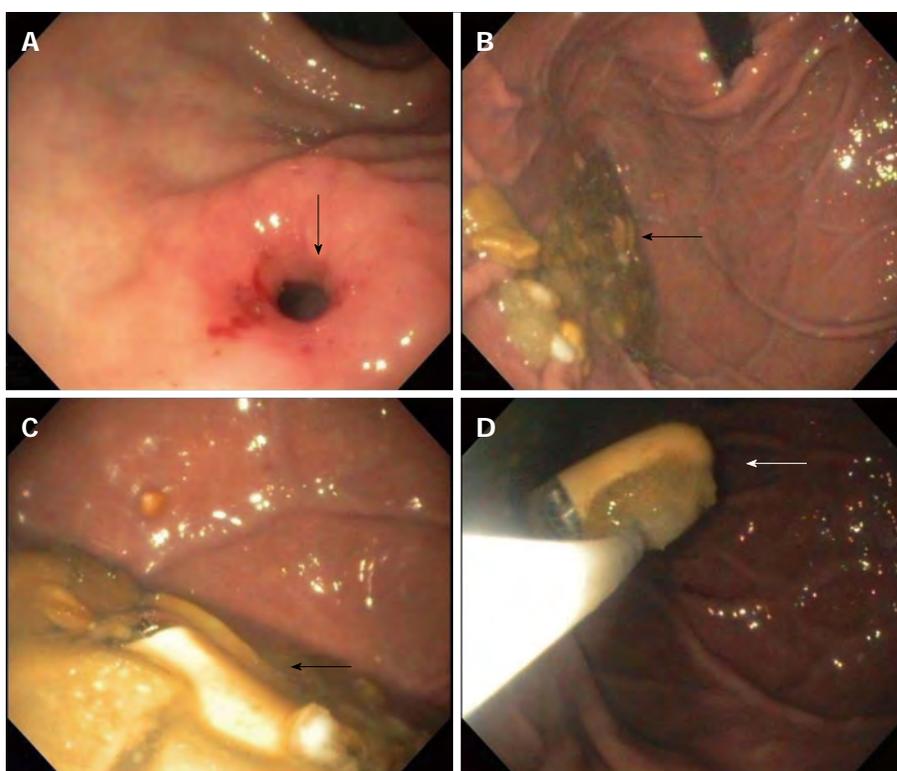
The PillCam<sup>®</sup> capsule (Given Imaging, Yoqneam, Israel) is 11 mm in diameter and 26 mm in length. Although VCE is generally regarded as safe<sup>[3,4]</sup>, capsule retention is recognized as a major complication of the procedure, potentially leading to bowel obstruction requiring its surgical removal. The 4<sup>th</sup> International Conference on Capsule Endoscopy (ICCE) in 2005 defined capsule retention as “having a capsule endoscope remain in the digestive tract for a minimum of 2 wk”. It was further defined as a “capsule remaining in the bowel lumen unless directed medical, endoscopic, or surgical intervention was instituted”<sup>[5]</sup>. The ICCE consensus did not set a time limit for removing retained capsules. In fact, asymptomatic retention and a possibility of reversing the cause of obstruction by directed medical management of the underlying condition may permit cautious non-surgical observation in select cases. Alternatively, double balloon enteroscopy may be helpful in removing retained capsule. The exact incidence of retained capsules varies widely from 0%<sup>[6]</sup> to 13%<sup>[7]</sup>, depending on the indication for the study. Patients with obscure GI bleeding are considered low risk, in contrast to patients with active Crohn’s disease or suspected small bowel obstruction where higher incidence of capsule retention was noted<sup>[7,8]</sup>. Careful history taking and small bowel radiologic evaluation will lead to anticipation of a potential obstruction, and use of biodegradable patency capsule may effectively assist in its precise localization. In general, a capsule retention requiring surgical intervention occurs at a rate of 0.75%<sup>[9]</sup>, ultimately leading to correct diagnosis of the underlying pathology.

Review of the medical literature to date shows that the duration of asymptomatic capsule retention varies greatly from few weeks to several years. Anatomically, the video capsule is most often retained in the ileum<sup>[5,10]</sup>. Until now, the longest reported case was 38 mo in a patient with small bowel Crohn’s disease<sup>[8]</sup>. To the best of our knowledge, our report describes the first case of a retained capsule in the stomach for over three years. It is also the first case report of adult hypertrophic pyloric stenosis diagnosed with video capsule endoscopy or a CT scan. In addition, we postulate that the physiologic worsening of our patient’s symptoms over last three years was due to the added degree of gastric flow obstruction secondary to intermittent proximal “plugging” of the pyloric channel with a retained video capsule.

Hypertrophic pyloric stenosis (HPS) is a rare cause of gastric outlet obstruction. Infantile HPS is suspected in a neonate with projectile non-bilious vomiting and weight loss, and occurs in 0.2%-0.5% of births. Classically, “ol-



**Figure 1 Radiologic imaging.** A: Retained video capsule on abdominal roentogram (arrow); B: Barium upper gastrointestinal series (arrow) shows pronounced delay in gastric emptying with a narrowed slightly elongated pylorus and a distended gastric antrum; C: Computed enterography of the abdomen shows retained video capsule, distended antrum of the stomach, and abnormally dense, eccentric, and significantly narrowed pylorus (arrow).



**Figure 2 Endoscopic findings.** A: Eccentric hypertrophic pyloric stenosis (arrow); B: Retroflexed view of the gastric fundus shows freely floating 3 years old video capsule in pool of retained semi-digested food particles (arrow); C: Close up view of retained capsule (arrow); D: Endoscopic retrieval with Roth Net (arrow).

ive sign” of a prominent pyloric muscle is palpated on physical examination and transabdominal ultrasound is diagnostic. Surgical intervention provides excellent prognosis. In contrast, adult HPS is a rare disorder that is further characterized as primary (idiopathic) or secondary (in association with peptic ulcer disease, malignancy, or hypertrophic gastropathy). To date, only over 200 cases of primary adult HPS have been described in the medical literature<sup>[11]</sup>, although its prevalence is likely under-reported. Males are more likely to be affected, and although affected patients range from 14 to 85 years of age, it is mostly diagnosed in fourth and fifth decades of life<sup>[12]</sup>. It has been traditionally accepted that primary

idiopathic adult HPS is likely a delayed presentation of a pediatric subclinical HPS. Histologically, there is marked hypertrophy and hyperplasia of the circular pyloric muscle. Grossly, pyloric channel greater than 1 cm in length and over 8 mm of muscular wall thickness is considered hypertrophic<sup>[13]</sup>. Expected diameter of normal pyloric orifice ranges from 1.2 to 1.5 cm<sup>[12]</sup>. Endoscopic findings may include “cervix sign” - a fixed narrowed pylorus with smooth borders that may preclude normal duodenal intubation with a standard gastroscope. Pyloric channel may also be eccentric in relation to the antrum with slight tenting towards lesser curvature. Upper GI series may demonstrate “Kirklín’s sign” - a mushroom-like

deformity at the base of the duodenal bulb<sup>[11]</sup>. Clinically, patient may present with early satiety, abdominal fullness, nausea, epigastric discomfort and eructation. Vomiting of undigested foods may provide symptomatic improvement. Due to outlet obstruction, a gastric scintigraphy may show delayed emptying, leading to a common misdiagnosis of gastroparesis and a delay in effective treatment. Management of symptomatic adult idiopathic HPS includes endoscopic balloon dilatation<sup>[14]</sup>, surgical pyloroplasty or Billroth I or II resection<sup>[11]</sup>. Potential benefit of Botulinum toxin injection in the pyloric sphincter may be of clinical interest, although its use in HPS has never been reported.

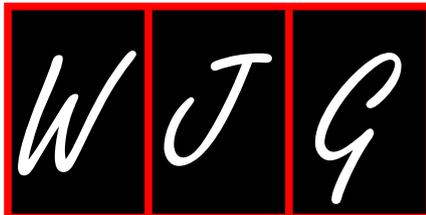
In conclusion, we report a first case in the medical literature of idiopathic adult HPS diagnosed by unusual finding of a retained video capsule endoscope in the stomach of the patient for over 3 years. This case raises a reasonable speculation that the use of a standard patency video capsule may, in certain cases, be applied to establish a diagnosis of occult HPS, differentiating it from an idiopathic gastroparesis, in which normal pyloric opening will eventually permit passage of the capsule into the small bowel. Permanent gastric retention of an 11 mm capsule may be detected by an abdominal roentogram and the device may be easily retrieved with an endoscope. It should be also noted that, specific to primary adult HPS, as large free floating capsule follows normal food bolus propagation it may effectively block an already compromised pyloric channel proximally, thus causing transient gastric outlet obstruction and appreciable worsening of patient's chronic symptoms. GI community should be aware of unique findings of capsule endoscopy in patients with HPS and use them to our advantage in arriving to correct diagnosis. In addition, advances in radiologic imaging have brought an important tool in CT enterography that, with better spatial resolution, may replace upper GI series as a test of choice in diagnosing primary HPS. Finally, this case raises clinical awareness of adult HPS, an uncommon medical condition that may mimic idiopathic gastroparesis, and importantly points out the need to vigilantly review antecedent workup in a complex patient with chronic GI symptoms to arrive to correct diagnosis.

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## An ironic case of liver infections: *Yersinia enterocolitis* in the setting of thalassemia

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### Abstract

A 49 years old Vietnamese male with a history of thalassemia, presented with gastrointestinal symptoms and signs of hemolysis. He was diagnosed with *Yersinia enterocolitis*. *Yersinia* is a gram-negative rod that most frequently occurs in children especially during the winter months. In the current case, the bone marrow biopsy showed hemophagocytosis along with positive cultures for *Yersinia*. The microorganism likely triggered hemophagocytosis. This syndrome, also known as, hemophagocytic lymphohistiocytosis, is defined by fever for more than 7 d, cytopenia of two or more cell lines, hemophagocytosis, hepatitis, serum ferritin greater than 500, jaundice, lymphadenopathy, and hepatosplenomegaly. This disorder can be either familial or secondary to a strong immunologic activation. Both have an overwhelming activation of T-cells and macrophages.

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**Key words:** *Yersinia*; Enterocolitis; Bone marrow; Liver

biopsy; Thalassemia; Hemophagocytic lymphohistiocytosis

**Core tip:** In the current case, the bone marrow biopsy showed hemophagocytosis along with positive cultures for *Yersinia*. The microorganism likely triggered hemophagocytosis. This syndrome, also known as, hemophagocytic lymphohistiocytosis, is defined by fever for more than 7 d, cytopenia of two or more cell lines, hemophagocytosis, hepatitis, serum ferritin greater than 500, jaundice, lymphadenopathy, and hepatosplenomegaly. This disorder can be either familial or secondary to a strong immunologic activation. Both have an overwhelming activation of T-cells and macrophages.

Selsky N, Forouhar F, Wu GY. An ironic case of liver infections: *Yersinia enterocolitis* in the setting of thalassemia. *World J Gastroenterol* 2013; 19(37): 6296-6298 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6296.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6296>

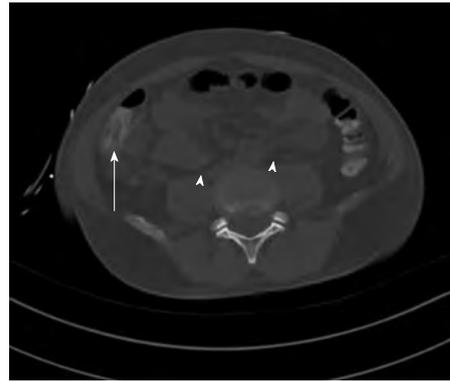
### INTRODUCTION

*Yersinia* is a gram-negative rod that most frequently occurs in children especially during the winter months. Transmission is largely food and waterborne. Pigs are frequently colonized with strains that cause human illness. Incubation typically lasts 2-6 d followed by a diarrheal period that can last up to three weeks. Symptoms include nausea, vomiting, and abdominal pain. Most strains of *Yersinia* grow poorly in typical agar solutions because the bacteria lack a mechanism for the efficient uptake of iron. Individuals who have iron overload due to either primary or secondary hemochromatosis are at increased risk of infection, and are also at higher risk to develop severe infections. Complications of severe infection can include diffuse ulcerating ileitis and colitis, intussusception, perforation, toxic megacolon, cholangitis, mesenteric vein

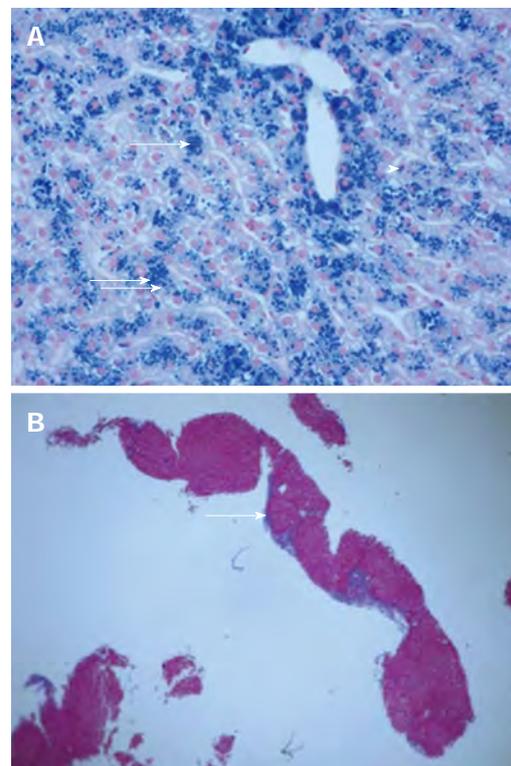
thrombosis, and hemophagocytic lymphohistiocytosis. Post-infectious complications include erythema nodosum and reactive arthritis. Treatment, reserved only for severe systemic infections, should consist of a 3<sup>rd</sup> generation cephalosporin and gentamicin for 3 wk. Genetic studies on this patient showed a loss of three alpha globin genes indicating the presence of Hb H disease. This lack of alpha globin causes a relative increase in the number of beta globin chains which can aggregate to form unstable tetramers. The tetramers have abnormal oxygen dissociation curves reflected in poor delivery of oxygen to the periphery, as well as precipitation of the hemoglobin tetramers as Heinz bodies. These precipitants can induce phagocytosis of red blood cells and a chronic hemolytic anemia which in turn leads to an increase in serum hep- cidin levels with resultant elevated iron transport across the gut mucosa. Over time, this leads to a systemic iron overload which can also be exacerbated iatrogenically by blood transfusions.

## CASE REPORT

A 49-year-old Vietnamese male, with a history of malaria 27 years ago was well until 5 d prior to admission when he developed dark urine associated with fevers, chills, and night sweats. This was followed by non-bloody diarrhea, and right upper quadrant abdominal pain as well as nausea and non-bloody vomiting. He denied any IV drug abuse, sick contacts, or travel history. He drank alcohol socially, but not to excess. On physical exam, he had a temperature of 104.3 °C, Blood pressure of 102/59 mmHg, heart rate of 100 beats/min, and saturation of 92% on room air. Generally, he was pale, diaphoretic, and sclerae were icteric. Abdominal examination revealed some right upper quadrant tenderness, but no rebound or guarding, and no hepatosplenomegaly. He had no rashes or stigmata of chronic liver disease. His laboratory studies showed a hemoglobin of 6.1 (13.8-18.0) g/dL with an MCV of 58 (80-100) fL, a white cell count of 10.6 (4.8-10.5) 10<sup>3</sup>/μL, and a platelet count of 75 (150-400) 10<sup>3</sup>/μL. Aspartate aminotransferase and alanine aminotransferase were 168 and 160 (5-40 and 7-56) U/L, respectively with a total bilirubin of 3.3 (0.3-1.9) mg/dL, and a direct bilirubin of 1.1 (0-0.3) mg/dL. Haptoglobin was < 15 (41-165). A peripheral smear demonstrated marked anisopoikilocytosis with schistocytes and target cells. Iron saturation was initially normal, 29%, with a ferritin of 6148 (12-300) mg/dL. Subsequent testing revealed persistently high iron saturation, 80%, and ferritin levels > 1000 mg/dL. Glucose and electrolytes were normal. Computerized tomography (CT) of the abdomen showed a normal biliary tree, proximal ascending colon mural thickening with surrounding adenopathy and pericolic stranding as well as bilateral pleural effusions (Figure 1). Stool, blood, and bone marrow cultures were all positive for *Yersinia enterocolitica*. The patient was positive for HBsAg with a viral load of 270000 IU. Genetic testing revealed mutations of three alpha globin genes making a diagnosis of alpha thalassemia (Hb H). A liver biopsy showed 3+ iron in hepatocytes

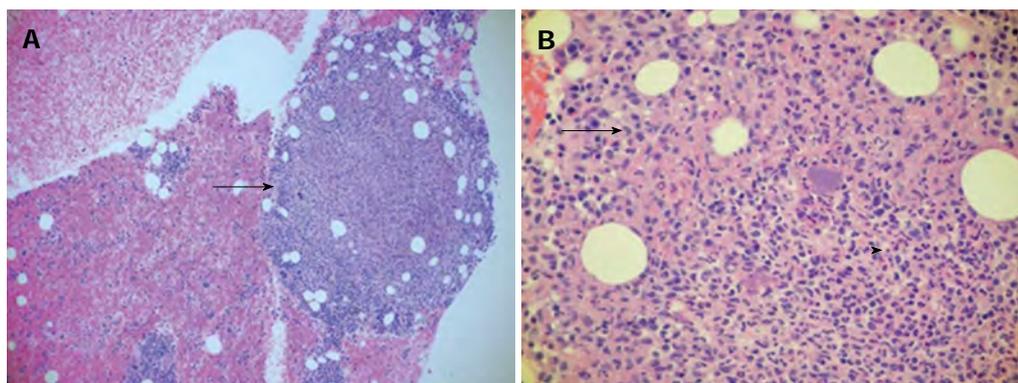


**Figure 1** A computer tomography of the abdomen without contrast performed on the day of admission. There was moderate mural thickening of the proximal ascending colon (arrow) with surrounding adenopathy and mild pericolic stranding. Also visible are mesenteric, pericolic and retroperitoneal lymph nodes (arrowheads) with the largest measuring 1.6 cm in short axis in the right pericolic region.



**Figure 2** Liver biopsy. A: There is marked, 3+, accumulation of iron primarily in the hepatocytes (arrows), but also in Kupfer cells (arrow head), and bile duct epithelium in association with moderate lobular hepatitis (Prussian Blue stain for iron, × 400); B: There is increased fibrosis with focal portal-to-portal and occasional central-portal septum formation (arrow) indicating progression towards early cirrhosis (Masson Trichrome stain, × 40).

with a portal to central gradient (Figure 2A), and chronic inflammation with early septum formation (Figure 2B). Bone marrow biopsy revealed iron overload, and a granuloma (Figure 3A), and hemophagocytic lymphohistiocytosis (Figure 3B)<sup>[1]</sup>. The patient was started on piperacillin/tazobactam/gentamicin and transfused to a hemoglobin level of 10 g/dL with rapid clinical improvement. He was discharged on a 3-wk course of oral ciprofloxacin, and a



**Figure 3 Bone marrow biopsy.** A: A necrotizing granuloma (arrow) with trilineage maturation and markedly increased iron storage [hematoxylin and eosin (HE) stain, ×100]; B: An area of necrosis (arrowhead) with erythrophagocytosis typical, but not diagnostic of *Yersinia* infection (HE stain, ×400).

follow up CT of the abdomen showed resolution of the bowel thickening and disappearance of the fat stranding. In addition, he was treated with oral deferasirox (Exjade) and entecavir. His ferritin level decreased to 842 by 12 wk. His liver enzyme levels returned to normal, and his HBV viral load became undetectable.

## DISCUSSION

In the current case, the bone marrow biopsy showed hemophagocytosis along with positive cultures for *Yersinia*. The microorganism likely triggered hemophagocytosis<sup>[2]</sup>. This syndrome, also known as, hemophagocytic lymphohistiocytosis, is defined by: fever for more than 7 d, cytopenia of two or more cell lines, hemophagocytosis, hepatitis, serum ferritin greater than 500, jaundice, lymphadenopathy, and hepatosplenomegaly. This disorder can be either familial or secondary to a strong immunologic activation. Both have an overwhelming activation of T-cells and macrophages.

In this patient, the chronic anemia due to thalassemia, or anemia in combination with the hepatitis B caused a secondary hemochromatosis<sup>[3]</sup>. This increased the risk of

*Yersinia* infection, and likely was responsible for the severity of the systemic infection<sup>[4]</sup>.

This patient will need iron chelation therapy and close monitoring for development of hepatocellular carcinoma because of the heightened risk with his coexisting HBV infection.

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## A case of plasmablastic lymphoma of the liver without human immunodeficiency virus infection

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### Abstract

Plasmablastic lymphoma (PBL) is a very rare B-cell lymphoproliferative disorder with an aggressive clinical behavior that recently characterized by the World Health Organization. Although PBL is most commonly observed in the oral cavity of human immunodeficiency virus (HIV)-positive patients, it can also be observed at extra-oral sites in HIV-negative patients. Epstein-Barr virus (EBV) may be closely related to the pathogenesis of PBL. PBL shows different clinicopathological characteristics between HIV-positive and -negative patients. Here, we report a case of PBL of the liver in a 79-year-old HIV-negative male. The patient died approximately 1.5 mo after examination and autopsy showed that the main lesion was a very large liver mass. Histopathological examination of the excised lesion showed large-cell lymphoma with plasmacytic differentiation diffusely infiltrating the liver and involving the surrounding organs. The neoplastic cells were diffusely positive for CD30,

EBV, Bob-1, and CD38. The autopsy findings suggested a diagnosis of PBL. To our knowledge, the present case appears to be the first report of PBL with initial presentation of the liver in a patient without HIV infection.

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**Key words:** Plasmablastic lymphoma; Human immunodeficiency virus-negative; Normal liver; Pathogenesis; Immunohistochemistry

**Core tip:** Plasmablastic lymphoma (PBL) is a rare B-cell lymphoma. Although PBL is observed in the oral cavity of human immunodeficiency virus (HIV)-positive patients, it can also be observed at extra-oral sites in HIV-negative patients. We present a case of PBL of the liver in a 79-year-old HIV-negative male. Histopathological examination showed large-cell lymphoma with plasmacytic differentiation diffusely infiltrating the liver and involving the surrounding organs. The neoplastic cells were diffusely positive for CD30, Epstein-Barr virus, Bob-1, and CD38. The present case appears to be the first report of PBL with initial presentation in the liver in a patient without HIV infection.

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### INTRODUCTION

Plasmablastic lymphoma (PBL) is a distinct, aggressive B-cell neoplasm that shows a diffuse proliferation of large neoplastic cells resembling B-immunoblasts with

an immunophenotype of plasma cells. PBL was initially described in 1997 as a rare subtype of diffuse large B-cell lymphoma (DLBCL) and has an aggressive clinical behavior arising in the oral cavity of human immunodeficiency virus (HIV)-infected individuals<sup>[1]</sup>. There have been several reports of PBL in HIV-negative patients, mainly at extra-oral sites including the stomach, small intestine and colon<sup>[2-6]</sup> but not in the liver. Interestingly, most cases of PBL occur without HIV infection in Japan and Korea, where the prevalence of HIV infection is low in comparison to Western countries<sup>[7,8]</sup>. In the present study, we describe the first case of liver PBL in a 79-year-old HIV-negative Japanese patient.

## CASE REPORT

A 79-year-old Japanese man was admitted to our hospital with the chief complaint of abdominal pain and icterus. Upon physical examination, jaundice was found to be prominent, which gradually worsened without relief of symptoms. Abdominal examination revealed the liver was palpable about 10 cm below the costal margin. The transaminase, gamma glutamyl transpeptidase, alkaline phosphatase, and bilirubin levels all notably exceeded normal values. An abdominal ultrasound showed dilatation of the intrahepatic bile ducts and a heterogeneous large mass that was 15 cm in diameter located in the right hepatic lobe. Radiographs and computed tomography (CT) of the chest, oral, and peri-oral sites showed no abnormalities, and no peripheral lymphadenopathy was observed. Serological tests to detect specific antibodies against hepatitis B and C virus were negative. The carcinoembryonic antigen and alpha-fetoprotein levels were normal, but soluble interleukin-2 receptor was high (5760 U/mL), and the patient tested negative for an anti-HIV antibody. The examination of the serum levels of Bence-Jones protein and rheumatoid factor was also negative. The patient's medical history included a gastrectomy for a gastric ulcer 20 years earlier and a hepatectomy for a liver abscess 5 years earlier. Plain (Figure 1A) and enhanced (Figure 1B) abdominal CT revealed a liver tumor and hepatomegaly with the same lesion present on ultrasonography (US).

Following examination, a US percutaneous-guided fine needle liver biopsy was performed. Histopathological examination of the biopsy smears showed a dense infiltrate composed of diffuse and proliferative large immunoblast cells with a typical morphological appearance of high-grade non-Hodgkin's lymphoma. However, we were unable to make a diagnosis due to poor immunostaining and a low number of specimens. During the detailed examination, CT and US revealed tumor progression with jaundice, despite continued percutaneous transhepatic biliary drainage.

Considering the high tumor burden and poor prognosis, the patient chose not to receive chemotherapy. He received palliative care and passed away due to multiple

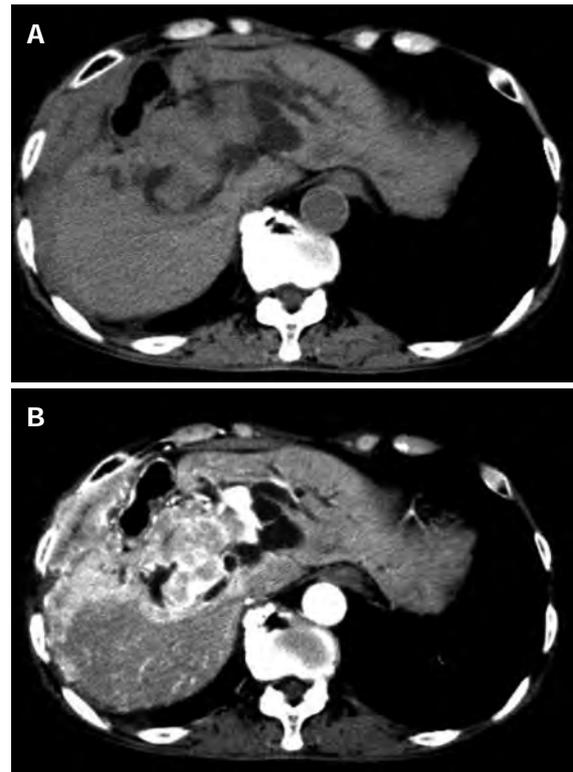
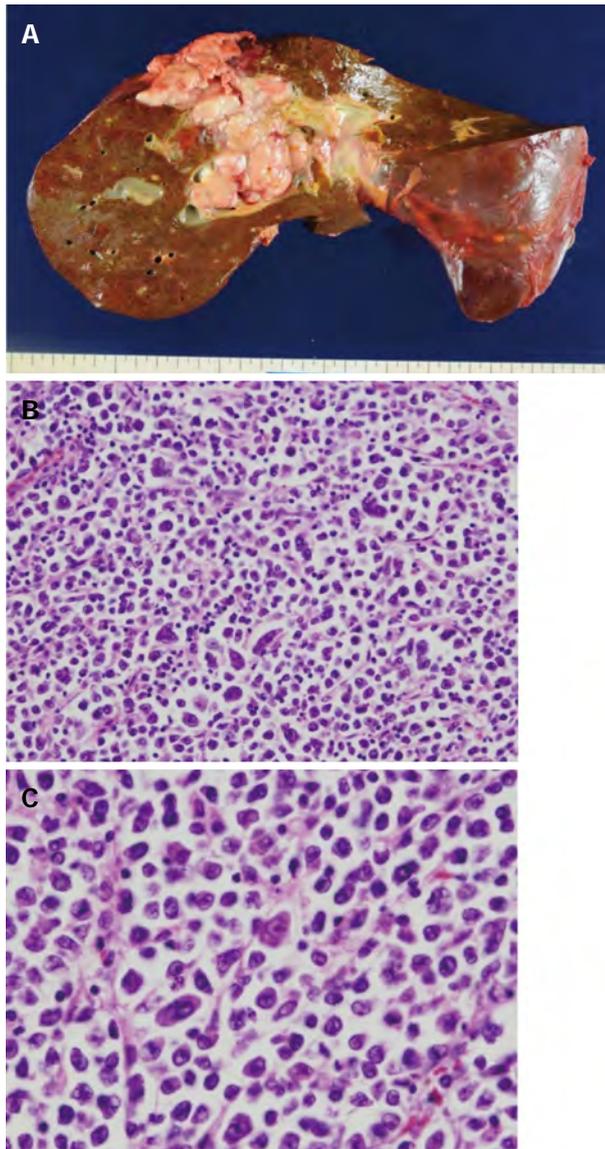


Figure 1 Computed tomography showing dilatation of the intrahepatic bile ducts and a heterogeneous large mass 15 cm in diameter located in the right hepatic lobe. A: Plain computed tomography (CT); B: Contrast-enhanced CT.

organ failure 1.5 mo after his initial clinical presentation. An autopsy was performed immediately after his death. Gross findings included a white, soft solid tumor of approximately diameter of 10 cm in the liver that involved the diaphragm and parietal peritoneum (Figure 2A). Histologically, large tumor cells with abundant basophilic cytoplasm were diffusely proliferative, and large nuclei were sporadically or centrally located and contained one or two conspicuous nucleoli in the central portion (Figure 2B). Specifically, the tumor cells had plasmablast- or immunoblast-like morphology, and there were almost no mature plasma cells present (Figure 2C). Additionally, binucleated or multinucleated tumor cells were scattered throughout the specimen, tumor cell proliferation in lymphatic vessels was conspicuous, and tumor cell invasion was found in some blood vessels.

Immunohistochemical examination revealed that the tumor cells were negative for B-cell markers CD20 (Figure 3A) and CD3 (Figure 3B) but positive for CD30 (Figure 3C), Epstein-Barr virus (EBV) (Figure 3D), and Bob-1 (Figure 3E). Although over 90% of the tumor cells were positive for Ki-67, they were negative for epithelial cell markers such as AE1/AE3, S100 protein, CD43, CD56, PAX-5, and human herpes virus (HHV)-8. Almost all tumor cells were highly positive for EBV virus-encoded RNA *in situ* hybridization (EBER-ISH). Based on these results, a diagnosis of PBL was made.



**Figure 2** Gross appearance of the primary tumor lesion at autopsy and histological appearance. A: A large tumor (diameter of 10 cm) is present in the liver. The cut section shows that the tumor is white and solid, with marked necrosis; B: Diffuse infiltration in the liver by monotonous large atypical lymphoid cells (HE staining; original magnification  $\times 200$ ); C: These atypical cells have an abundant basophilic cytoplasm, eccentrically located pleomorphic nuclei, and single, centrally located prominent nucleoli (HE; original magnification  $\times 400$ ).

## DISCUSSION

DLBCL is currently considered to be a heterogeneous group of rare tumors primarily occurring in the presence of HIV infection<sup>[9,10]</sup>. DLBCL with plasmablastic features has recently been categorized into the following subtypes: PBL of the oral cavity, PBL with plasmacytic differentiation, classic primary effusion lymphoma (PEL), extracavitary/solid PEL or HHV8-associated DLBCL, and anaplastic lymphoma kinase-positive DLBCL<sup>[10,11]</sup>.

PBL is a rare lymphoma accounting for approximately 2.6% of AIDS-related neoplasms<sup>[12]</sup> and typically occurs in the oral cavity of HIV-infected patients. Recently, however, PBL has been reported in patients without HIV

infection, and several cases have been reported in extra-oral locations, such as skin, subcutaneous tissue, stomach, anal mucosa, lung, and lymph node regions<sup>[3-6,13,14]</sup>. PBL has also been observed in immunocompromised individuals such as organ transplant patients and the elderly. PBL with HIV infection primarily affects men at a young age, while the clinical characteristics of PBL without HIV infection are typically old age with a slight male predominance<sup>[15]</sup>. This difference reflects the epidemiology of HIV infection. In addition, over one-third of all cases with PBL were first noted at extra-oral locations<sup>[16]</sup>. Interestingly, although the gastrointestinal tract has been observed to be the most common extraoral site (10.6%), the liver is a rare extra-oral location in PBL patients<sup>[16]</sup>. Only one case of liver PBL patient with HIV infection has been reported<sup>[17]</sup>.

PBL is a distinct, aggressive B-cell neoplasm that shows a diffuse proliferation of large neoplastic cells resembling B-immunoblasts with an immunophenotype of plasma cells. PBL cells predominantly exhibit the morphological features of plasmablasts/immunoblasts and are immunohistologically negative or slightly positive for B-cell markers such as CD20 and CD79a but positive for plasmacyte markers such as CD38 and CD138. PBL cells are highly reactive to the cell proliferation marker Ki67, and 2 in 3 patients carry an integrated EBV genome, while HHV8 is negative. EBER-ISH is highly positive in PBL cells; in particular, EBER-ISH has a positive predictive value of close to 100% in HIV-positive PBL patients with presentation in the oral cavity<sup>[18,19]</sup>.

Differential diagnosis is needed for poorly differentiated carcinomas and malignant melanomas, in addition to malignant lymphomas, such as anaplastic (plasmablastic) plasmacytoma (AP), Burkitt's lymphoma, and DLBCL-NOS<sup>[20]</sup>. Although a differential diagnosis of AP is especially difficult because of tumor cell morphology and immunohistochemical results that are similar to other subtypes, AP cases are usually preceded by a plasmacytoma such as multiple myeloma.

Because the positive value of EBER is significantly high in PBL patients, it has been suggested to use this value as a diagnostic tool<sup>[18]</sup>. However, some plasmacytomas with no signs of immune system abnormalities exhibit plasmablast-like morphology with a particularly high EBER-positive rate<sup>[21]</sup>. To differentiate the two diseases, it is important to use clinical findings (such as the presence of multiple myeloma and M protein) in addition to histopathological and immunohistological findings. The overall prognosis of PBL is poor<sup>[12,15]</sup>; however, the prognosis of PBL in HIV-positive patients has improved with the enhanced management of HIV symptoms, but it has worsened in HIV-negative patients, which include a large elderly population<sup>[7,15]</sup>. MYC/IgH translocation is observed in 70% of EBER-positive PBL patients, and, interestingly, these patients have a notably worse PBL prognosis, presenting with an endemic Burkitt lymphoma-like phenotype and symptoms<sup>[22,23]</sup>.

The management of PBL is based mainly on early

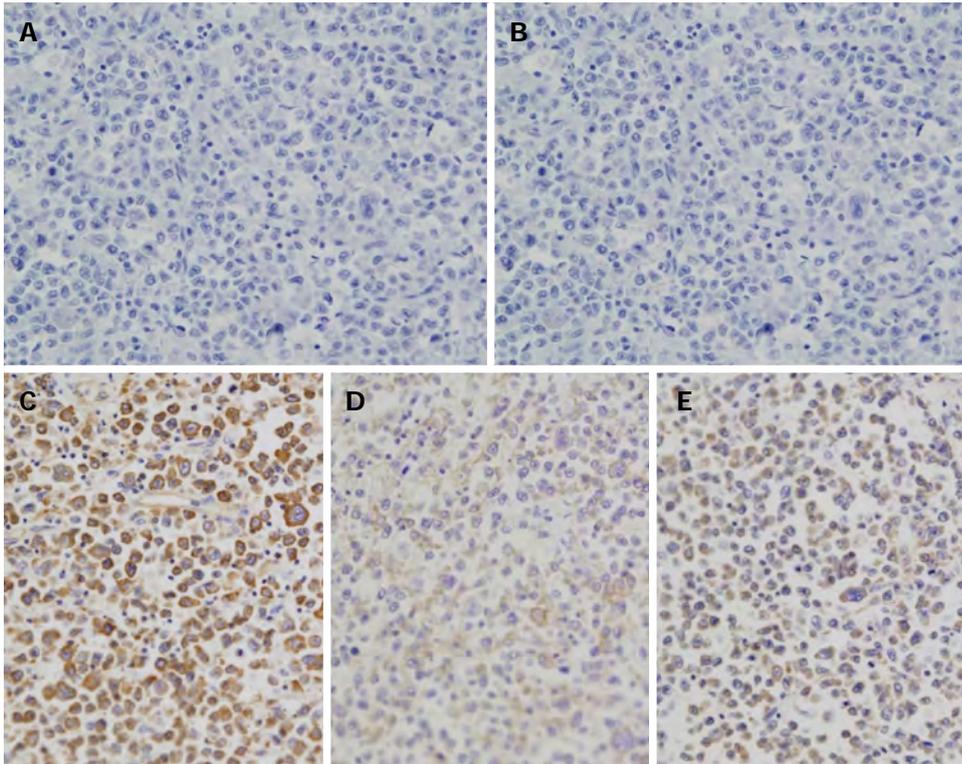


Figure 3 Immunohistochemical staining showing tumor cells with a negative expression of CD20 (A) and CD3 (B), positive expression of CD30 (C), Epstein-Barr virus (D), and Bob-1 (E).

aggressive chemotherapy. Cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) and CHOP-like regimens are commonly used with a good overall response rate but have a high relapse rate and poor overall survival<sup>[24]</sup>.

In our case, no primary carcinoma was detected in any organs at autopsy. In cases of malignant melanoma, tumor cells are immunohistochemically positive for melanosomes and S-100 protein, and Burkitt's lymphomas expresses LCA, CD20, and CD79a. A differential diagnosis between PBL and plasmacytoma is often difficult without histological examination. Because PBL is composed almost entirely of blast cells, plasmacytoma typically consists of mature plasma cells. Therefore, the morphology and immunophenotype of both tumor cells, as well as clinical features, are essential for an accurate diagnosis of PBL.

The condition of the patient rapidly deteriorated because we failed to provide proper treatment due to the unsuccessful initial histopathological examination of the liver biopsy. If biopsy specimens show similar pathological features to those presented in this study, a differential diagnosis of PBL and an immunohistochemical analysis of CD138 and EBER are urgently needed. Although there is one reported case of PBL originating in the liver<sup>[17]</sup>, to our knowledge, this is the first case report of PBL that occurred in the liver without HIV infection. Because extra-oral PBL can occur in HIV-negative patients and has a poor prognosis, it should be included as a differential diagnosis in cases of suspected hepatic lymphoma.

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## Composite diffuse large B-cell lymphoma and classical Hodgkin's lymphoma of the stomach: Case report and literature review

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### Abstract

The combination of classical Hodgkin's lymphoma (cHL) and non-Hodgkin lymphoma coexisting in the same patient is not common, especially in one extranodal location. Here we present a rare case of composite diffuse large B-cell lymphoma (DLBCL) and cHL occurring simultaneously in the stomach of a 53-year-old female who presented with upper abdominal discomfort and gas pain. Surgery was performed and the disease was diagnosed pathologically as composite lymphoma of DLBCL and cHL using hematoxylin-eosin and immunohistochemical staining. Epstein-Barr virus (EBV) infection was not detected by *in situ* hybridization for EBV-encoded RNA or immunohistochemistry for EBV latent membrane protein-1. Polymerase chain reaction analysis from the two distinct components of the tumor demonstrated clonal immunoglobulin  $\kappa$  light chain gene rearrangements. The patient died approximately 11 mo after diagnosis in spite of receiving eight courses of the CHOP and two courses of the rituximab-CHOP (RCHOP)

chemotherapy regimen. This case report showed that the two distinct components, DLBCL and cHL, appeared to originate from the same clonal progenitor cell, and that EBV infection was not essential for transformation during the course of tumorigenesis.

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**Key words:** Composite lymphoma; Diffuse large B-cell lymphoma; Hodgkin's lymphoma; Stomach

**Core tip:** Classical Hodgkin's lymphoma (cHL) commonly manifests in lymph nodes whereas primary extranodal cHL in the gastrointestinal tract is very rare, and only single cases of primary gastric cHL have been reported in the literature. The combination of cHL and non-Hodgkin lymphoma (NHL) coexisting in the same patient is not common, especially in one extranodal location. The combination of cHL and NHL coexisting in the stomach is extremely rare. Here we present a case of composite diffuse large B-cell lymphoma and mixed cellularity cHL involving the stomach, and present a review of the literature.

Wang HW, Yang W, Wang L, Lu YL, Lu JY. Composite diffuse large B-cell lymphoma and classical Hodgkin's lymphoma of the stomach: Case report and literature review. *World J Gastroenterol* 2013; 19(37): 6304-6309 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6304.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6304>

### INTRODUCTION

Composite lymphoma (CL), which is defined as the coexistence of two or more morphologically and phenotypically distinct lymphoma types in a single anatomic organ or tissue, is unusual<sup>[1]</sup>. Almost all the primary stomach



Figure 1 Gastroscopy showing an irregular ulcer covered with white exudates (A) and multiple mucosal nodularities in the gastric corpus (B) and a circular ulcer in the gastric pyloric canal (C).

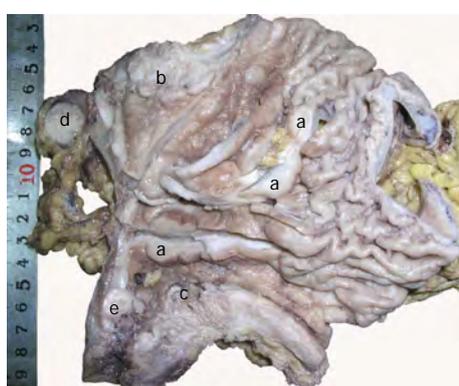


Figure 2 Macroscopic findings of the lesions. Multiple mucosal nodularities (a) and an ulcer (b) in the gastric corpus, a circular ulcer in the gastric pyloric canal (c), perigastric (d) and parapyloric (e) swollen lymph nodes.

lymphomas are non-Hodgkin's lymphoma (NHL), the majority of which are of B-cell origin, and mucosa-associated lymphoid tissue lymphoma and diffuse large B-cell lymphoma (DLBCL) account for over 90%<sup>[2]</sup>. Classical Hodgkin's lymphoma (cHL) commonly manifests in lymph nodes whereas primary extranodal cHL in the gastrointestinal tract is very rare, estimated at 0.025% of all cHL, and only single cases of primary gastric cHL have been reported in the literature<sup>[3]</sup>. The combination of cHL and NHL coexisting in the stomach is extremely rare. Here we present a rare case of composite DLBCL and mixed cellularity cHL involving the stomach, and present a review of the literature.

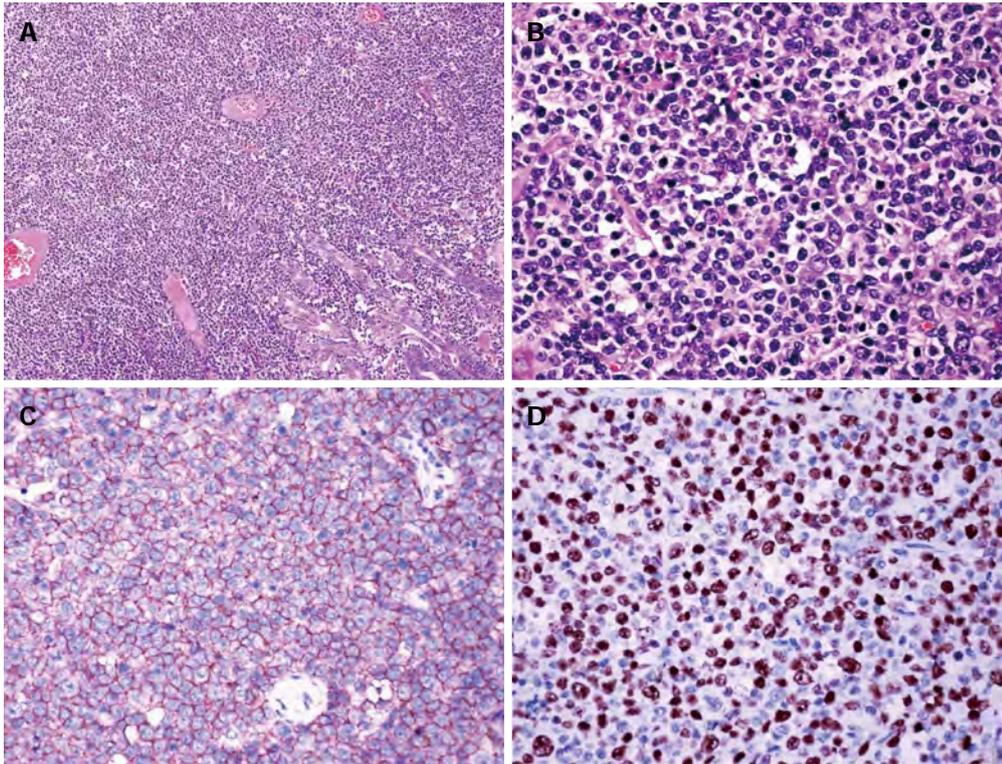
## CASE REPORT

A 53-year-old female presented with upper abdominal discomfort and flatulent pain for over an 8-mo period and her condition became gradually worse. She also described a substantial weight loss and anorexia over the preceding 6 mo. Computed tomography (CT) scans of the abdomen showed thickening of the wall in the gastric pylorus and gastric corpus, and uneven enhancement could be seen after intravenous administration of contrast agent. Gastroscopy revealed an irregular ulcer

covered with white exudates (Figure 1A) and multiple mucosal nodularities in the gastric corpus (Figure 1B). Another circular ulcer covered with white exudates and effusion was simultaneously found in the gastric pyloric canal (Figure 1C). Biopsy specimens were obtained from the two ulcers. A histologic diagnosis of small round cell malignant tumor, indicating lymphoma, was made. Routine blood examination showed hemoglobin 115 g/L, white blood cell count  $5.32 \times 10^9/L$ , neutrophils 52.3%, lymphocytes 19.6%, monocytes 12.1%. Serological testing demonstrated negativity for hepatitis B virus and human immunodeficiency virus infections. The lactate dehydrogenase level (185 U/L) was in the normal range. Abdominal ultrasonography and computed tomography scans of the chest did not show any other abnormalities. No superficial lymphadenopathy was noted.

The patient underwent distal stomach resection because of aggravated symptoms of obstruction. Grossly, the greater and lesser curvatures of the resected stomach measured 17.0 and 7.5 cm respectively. The gastric wall was diffusely thickened and multiple different sizes of mucosal nodularities ranging from 1 cm to 2.5 cm in diameter (Figure 2, a) and a well circumscribed ulcer measuring 4 cm  $\times$  3.5 cm  $\times$  0.5 cm (Figure 2, b) were identified in the gastric corpus. The gastric pyloric canal presented with increased thickness and stenosis, and a circular ulcer measuring 3 cm  $\times$  3 cm  $\times$  0.8 cm was also found (Figure 2, c). The cut surface of the neoplastic ulcers and mucosal nodularities were grey and soft. Perigastric (Figure 2, d) and parapyloric (Figure 2, e) swollen lymph nodes were identified. Selected tumor tissues were fixed in formalin and embedded in paraffin and cut into sections then stained with hematoxylin and eosin for routine histology. Additional sections of paraffin-embedded tissue were used for immunohistochemical staining and *in situ* hybridization analysis. Genomic DNA was isolated from CD30<sup>+</sup> Hodgkin and Reed-Sternberg (RS) cells and CD20<sup>+</sup> DLBCL cells by micromanipulation, and polymerase chain reaction (PCR) procedures were performed for analysis of immunoglobulin heavy and  $\kappa$  light chain rearrangements.

Microscopically, there were two morphologically and



**Figure 3** Diffuse large B-cell lymphoma of the stomach. A: Large lymphoid cells diffusely infiltration the gastric corpus wall (HE,  $\times 100$ ); B: Nucleoli and frequent mitotic figures (HE,  $\times 400$ ); C: Neoplastic cells diffusely positive for CD20 (immunoperoxidase stain,  $\times 400$ ); D: Nuclear proliferation rate as assessed by Ki-67 staining was approximately 80% (immunoperoxidase stain,  $\times 400$ ). HE: Hematoxylin and eosin.

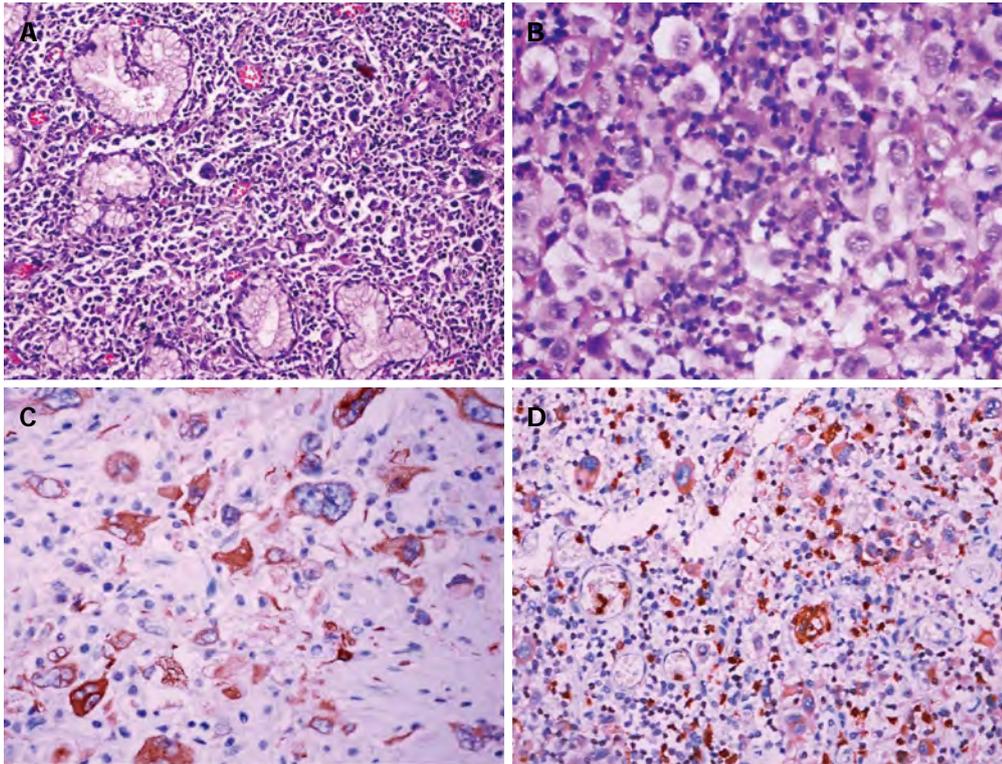
immunophenotypically distinct components in different locations of the stomach. The ulcer and multiple mucosal nodularities in the gastric corpus exhibited a homogeneously uniform population of large lymphoid cells infiltration all layers of the gastric wall (Figure 3A). The nuclei were round or multilobated, with finely dispersed chromatin and evident nucleoli. Frequent mitotic figures were noted (Figure 3B). The neoplastic cells showed uniform expression of CD45, CD20 (Figure 3C), CD79a, Pax-5, MUM1, and absence of CD3, Bcl-6 and CD10. The nuclear proliferation rate as assessed by Ki-67 staining was approximately 80% (Figure 3D). Additional immunohistochemistry displayed tumor cells negative for cytokeratin, CD30, CD15 and other T-cell antigens. The ulcer in the gastric pylorus showed typical mixed lymphocyte, eosinophil granulocyte and neutrophil granulocyte infiltration with fibrosis (Figure 4A), and contained numerous large atypical lymphoid cells, including Hodgkin and RS cells (Figure 4B). The Hodgkin and RS cells were positive for CD30 (Figure 4C), CD15 (Figure 4D), MUM1 and Oct-2, and weakly positive for Pax-5, but negative for CD45, CD20, CD79a, CD3, CD10 and BOB.1. Interestingly, the perigastric and parapyloric swollen lymph nodes were infiltrated by tumor cells of DLBCL and cHL, respectively. Neither cell population showed markers of Epstein-Barr virus (EBV) infection by *in situ* hybridization for EBV-encoded RNA or immunohistochemistry for EBV latent membrane protein-1. On the basis of these morphologic and immunohisto-

chemical characteristics, the pathological diagnosis of composite DLBCL and mixed cellularity cHL was made. PCR analysis from the two distinct components of the tumor demonstrated clonal immunoglobulin  $\kappa$  light chain gene rearrangements (Figure 5).

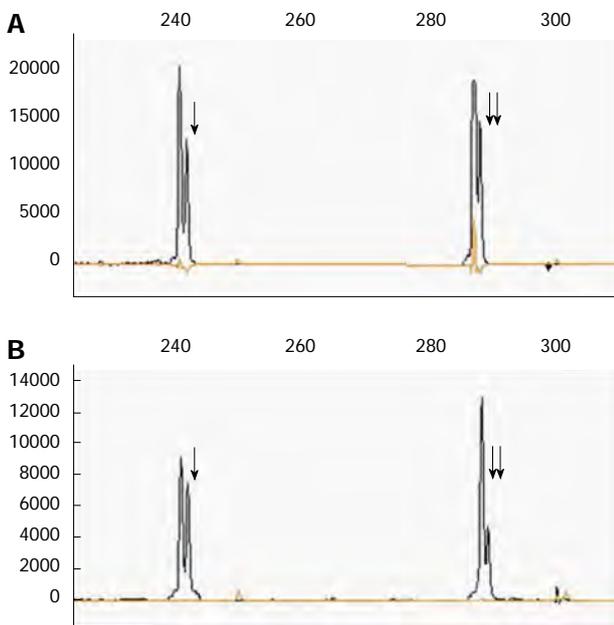
After surgery, the patient was treated with eight courses of a standard CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy regimen, after which she showed an excellent response with normal brain, thoracic and abdominal CT scans. Unfortunately, repeat CT scans and ultrasonography revealed tumor recurrence with abdominal tumor load 7 mo after chemotherapy. Then the patient received a further two cycles of rituximab-CHOP (RCHOP) chemotherapy. Unfortunately, she died of multiple organ failure due to lymphoma recurrence on the 11<sup>th</sup> postoperative month. An autopsy was not performed.

## DISCUSSION

The concept of CL was first put forward by Custer<sup>[4]</sup> to explain the occurrence of more than one histological type of lymphoma in the same patient. In the study of more than 1000 cases for the International Working Formulation for NHL, the incidence of CL varied between 1% and 4.7%<sup>[5]</sup>. cHL and NHL are morphologically and clinically distinct neoplasms. The combination of cHL and NHL coexisting in the same tissue is rare and much more uncommon than other combinations<sup>[3]</sup>. According



**Figure 4 Classical Hodgkin's lymphoma of the stomach.** A: Mixed lymphocyte, eosinophil granulocyte and neutrophil granulocyte infiltrating the gastric pyloric canal wall (HE,  $\times 200$ ); B: Hodgkin and Reed-Sternberg (RS) cells are present (HE,  $\times 400$ ); C, D: Hodgkin and RS cells positive for CD30 and CD15, respectively (immunoperoxidase stain,  $\times 400$ ). HE: Hematoxylin and eosin.



**Figure 5 Polymerase chain reaction analysis from the two distinct components of the tumor demonstrated clonal immunoglobulin  $\kappa$  light chain gene rearrangements.** The asterisks indicate two peaks representing the rearranged polymerase chain reaction products from position 241 bp (arrow) and 281 bp (double arrows) regions of immunoglobulin  $\kappa$  light chain gene, respectively. A: DNA from the dissected diffuse large B-cell lymphoma component. B: DNA from the dissected classical Hodgkin lymphoma component.

to our literature review, a total of 10 cases, including the present case, reported a combination of DLBCL and cHL within the same site simultaneously<sup>[6-13]</sup>. The clinical data of all previously published cases of composite DLBCL and cHL are listed in Table 1. In these cases, there were five cases of combination of DLBCL and cHL in the lymph nodes, three cases in the stomach, one case in the small intestine and one case in the anterior mediastinum, indicating that the gastrointestinal tract is the most common extranodal site involved in this kind of composite lymphoma. Prochorec-Sobieszek *et al*<sup>[9]</sup> firstly reported localized gastric DLBCL and cHL as secondary neoplasms in two patients with chronic lymphocytic leukemia. To the best of our knowledge, this is the first case report of composite DLBCL and cHL coexisting in the stomach with no history of lymphoma or leukemia.

The pathogenesis of CL is inconclusive. Viral infections, genetic susceptibility, genetic mutations, and immune suppression are CL pathogenic factors. However, no single definite mechanism has been proposed to explain the pathogenesis of different types of CL as the etiology is variable, complex and differs according to the types of lymphomas involved<sup>[14-16]</sup>. In general, cHL is associated with EBV; on the other hand, NHL is infrequently associated with EBV. When cHL and NHL are present in the same anatomic site, there is a higher correlation with the presence of EBV in both lymphoma

**Table 1 Composite diffuse large B-cell lymphoma and classical Hodgkin's lymphoma: A review of the literature**

Ref.	Gender/age (yr)	Organ	EBV infection	Treatment	Follow up time	Status
Paulli <i>et al</i> <sup>[6]</sup>	Male/37	Supra-clavicular lymph nodes	NA	8 cycles of pro-MACE-CytaBOM chemotherapy	23 wk	ANED
Bellan <i>et al</i> <sup>[7]</sup>	Female/29	Cervical lymph nodes	NA	MACOP-B chemotherapy for 8 wk and autologous stem cells transplant	30 mo	ANED
Rosenquist <i>et al</i> <sup>[8]</sup>	Female/74	Inguinal lymph nodes	+	6 cycles of CHOP chemotherapy	12 wk	ANED
Prochorec-Sobieszek <i>et al</i> <sup>[9]</sup>	Male/67	Stomach	+	6 courses of CHOP chemotherapy	22 wk	ANED
	Male/76	Stomach	+	6 courses of cyclophosphamide and cladribine chemotherapy	14 wk	DOD
Huang <i>et al</i> <sup>[10]</sup>	Male/56	Small intestine	+	Lesion resected	6 d	DOD
Miyagaki <i>et al</i> <sup>[11]</sup>	Male/75	Axillary lymph nodes	+	6 courses of RCHOP chemotherapy	3 yr	DOD
Yu <i>et al</i> <sup>[12]</sup>	Female/37	Anterior mediastinum	-	6 courses of CHOP chemotherapy and 23 times radiotherapy	33 wk	ANED
Bautista-Quach <i>et al</i> <sup>[13]</sup>	Female/6	Multiple lymph nodes	+	Combined chemotherapy (program unknown)	17 wk	DOD
Wang <i>et al</i> (present case)	Female/53	Stomach	-	Lesion resected and 8 courses of CHOP and 2 courses of RCHOP chemotherapy	45 wk	DOD

NA: Not available; +: Positive; -: Negative; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; RCHOP: Rituximab-CHOP; ANED: Alive with no evidence of disease; DOD: Died of disease.

cells than when two lymphomas occur at different times and/or at different sites. If the two components demonstrate positivity for EBV, it would be suggested that a commonly infected progenitor cell might be responsible for both lymphomas<sup>[17,18]</sup>. From assessment of the data in Table 1, composite DLBCL and cHL often showed EBV positivity, suggesting an origin from a commonly EBV-infected progenitor cell; however, two components from two cases including our patient were all negative for EBV, indicating EBV infection did not seem to be the primary event in this tumorigenesis.

Lymphoma, generally, is defined as monoclonal proliferation of lymphocytes (T cell, B cell or natural killer cell). Coexistence of DLBCL and cHL in the same anatomic location has been reported occasionally; studies using molecular techniques have proved that they may be clonally related (*i.e.*, derived from the same lymphoid progenitors) or not related (*i.e.*, different lymphoid progenitors)<sup>[7-10]</sup>. Controversy about this issue may reflect the lack of a full understanding of the pathogenesis of these lymphomas or the heterogeneity of Hodgkin lymphoma and NHL. In the present case, identical immunoglobulin  $\kappa$  light chain gene rearrangements were seen in the two distinct components, indicating that both components, despite their distinctly different morphologic features and immunophenotype, were indeed derived from the same clone. Thus, DLBCL and cHL coexisting in the stomach in our case can be considered a true CL with two distinctive presentations.

The pathological differential diagnosis of composite DLBCL and cHL in the present case mainly included cHL transformation to DLBCL, anaplastic variant of DLBCL, T cell/histiocyte-rich large B-cell lymphoma (THRLBCL), anaplastic large cell lymphoma (ALCL) and grey zone lymphoma. The characteristic morphology and immunophenotype of the tumor cells in conjunction with clinical features aid in the differential diagnosis. cHL

transformation to DLBCL may be differentiated from the current case as the two distinct lymphoma occurred simultaneously within different sites of the stomach without a histological mixing zone. An anaplastic variant of DLBCL is characterized by large tumor cells with bizarre pleomorphic nuclei that may resemble Hodgkin and/or RS cells, and it may be differentiated according to its consistent immunological staining for B-cell markers, such as CD20 and CD79. THRLBCL is comprised of scattered, single, large B cells embedded in a background of T cells and a variable number of histiocytes. These large B cells may mimic Hodgkin and RS cells in cHL, but they express pan B-cell markers with no expression of CD15 and CD30. The "Hodgkin-like pattern" accounts for 3% of ALCL cases, which is characterized by morphological features mimicking nodular sclerosing cHL. CD15 expression is rarely observed and when present only a small proportion of the neoplastic cells are stained; however, Hodgkin and RS cells in cHL are always weakly positive for Pax-5, which is different from ALCL. The most presentation of grey zone lymphoma is a large anterior mediastinal mass with rare involvement of non-lymphoid organs. Some areas may more closely resemble DLBCL and others appear more like cHL. In cases that morphologically resemble cHL, uniform strong expression of CD20 and other B-cell markers and absence of CD15 would favor the diagnosis of grey zone lymphoma. Other histological differential diagnoses, including leukocytopenia, poorly differentiated carcinoma, sarcoma, reactive lymphoid proliferation and collision tumor should be cautiously considered<sup>[19]</sup>.

To conclude, we report a rare case of composite DLBCL and cHL involving the stomach and describe the histologic and immunophenotypic findings. The contribution of immunohistochemistry plays an important role in differential diagnosis. Using molecular techniques, we further proved that the two different components ap-

peared to originate from the same clonal progenitor cell, and that EBV infection was not essential for transformation during the course of tumorigenesis.

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## Giant biliary cystadenoma complicated with polycystic liver: A case report

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Author contributions: Yang ZZ collected the clinical data and wrote the paper; Li Y, Yan YH, Liu J and Xiao WD performed the operation; Li KF assisted in the reference search.

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### Abstract

Biliary cystadenoma (BCA) is a rare hepatic neoplasm. Although considered a benign cystic tumor of the liver, BCA has a high risk of recurrence with incomplete excision and a potential risk for malignant degeneration. Correct diagnosis and complete tumor excision with negative margins are the mainstay of treatment. Unfortunately, due to the lack of presenting symptoms, and normal laboratory results in most patients, BCA is hard to distinguish from other cystic lesions of the liver such as biliary cystadenocarcinoma, hepatic cyst, hydatid cyst, Caroli disease, undifferentiated sarcoma, intraductal papillary mucinous tumor, and hepatocellular carcinoma. Ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) may be necessary. They demonstrate intrahepatic cystic lesions with features such as mural nodules, varying wall thickness, papillary projections, and internal septations. Nevertheless, surgery is still the only means of accurate diagnosis. Definitive diagnosis requires histological examination following formal resection. We describe a 57-year-old woman initially diagnosed with polycystic liver who was subsequently diagnosed with giant intra-

hepatic BCA in the left hepatic lobe. This indicates that both US physicians and hepatobiliary specialists should attach importance to hepatic cysts, and CT or MRI should be performed for further examination when a diagnosis of BCA is suspected.

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**Key words:** Biliary cystadenoma; Diagnosis; Hepatic cysts; Ultrasound

**Core tip:** We present a case of a 57-year-old woman who was diagnosed with polycystic liver ten years ago. She had intermittent abdominal discomfort and pain in the past 2 years. Last month, she was admitted to our hospital, and underwent exploratory laparotomy with left hepatic lobectomy, right liver cyst fenestration, and cholecystectomy. She was then diagnosed with giant biliary cystadenoma complicated with polycystic liver.

Yang ZZ, Li Y, Liu J, Li KF, Yan YH, Xiao WD. Giant biliary cystadenoma complicated with polycystic liver: A case report. *World J Gastroenterol* 2013; 19(37): 6310-6314 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i37/6310.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i37.6310>

### INTRODUCTION

Biliary cystadenoma (BCA) is a rare hepatic neoplasm. Although considered a benign cystic tumor of the liver, BCA has a high risk of recurrence with incomplete excision and a potential risk for malignant degeneration. Correct diagnosis and complete tumor excision with negative margins are the mainstay of treatment. Unfortunately, due to the lack of presenting symptoms, and normal laboratory results in most patients, it is hard to distinguish BCA from other cystic lesions of the liver. Definitive diagnosis requires histological examination following for-

mal resection. We describe a 57-year-old woman initially diagnosed with polycystic liver who was subsequently diagnosed with a giant intrahepatic BCA in the left hepatic lobe. This indicates that importance should be attached to hepatic cysts in the ultrasound (US) examination, and computed tomography (CT) or magnetic resonance imaging (MRI) should be performed for further examination when a diagnosis of BCA is suspected.

## CASE REPORT

A 57-year-old woman was admitted to our hospital with intermittent abdominal discomfort and pain for almost 2 years. Discomfort and pain were not related to meals, defecation or change in position, and could be tolerated. Initially, she underwent an US examination of the abdomen at a local hospital. This revealed multiple small cysts in the liver, which caused no particular concern. She had not experienced diarrhea, nausea, vomiting, fever or chills since the onset of symptoms. However, she noticed that her abdominal girth appeared to be slowly increasing in size 1 mo ago, and repeat US examination at an outside institution showed a left hepatic multiloculated cystic mass measuring 21.0 cm × 9.1 cm × 13.6 cm with internal septations and multiple small cysts in the left liver lobe.

She visited our hospital for further treatment. On physical examination, it was significant for abdominal tenderness and the abdomen was distended, with a large, soft, non-mobile mass in the left half of the abdomen. The patient had no history of intravenous drug use or tattoos or body piercing, no history of excessive alcohol use or obesity, and no history of working with toxic chemicals. She had no prior history of surgery, medical illness, and no known allergies. She was not using any medication. There was no significant family history of biliary or liver diseases.

CT imaging performed at our institution demonstrated a left hepatic multiloculated cystic mass measuring 15.0 cm × 9.1 cm, occupying the majority of the upper abdomen, and normal liver structure had disappeared. The internal septations were visible and enhanced after intravenous administration of contrast medium. The mass cranially displaced the liver. The gallbladder and pancreas were compressed. Simultaneously, multiple sizes of hypoattenuating shadows without enhancement were seen in the right liver lobe (Figure 1). Abdominal MRI was ordered and revealed a large cystic tumor measuring approximately 18.0 cm × 9.0 cm originating from the left liver lobe (Figure 2). On T1-weighted imaging (T1WI), low signal intensity was apparent within the cystic spaces. On corresponding T2-weighted imaging (T2WI), the tumor was characterized by a medium-high intensity signal clearly delineated from the surrounding liver tissue with internal septal structures separating the fluid-filled spaces.

Laboratory tests were within normal limits, and serology for hepatitis B virus infection was negative, and serum carcinoembryonic antigen (CEA), carbohydrate

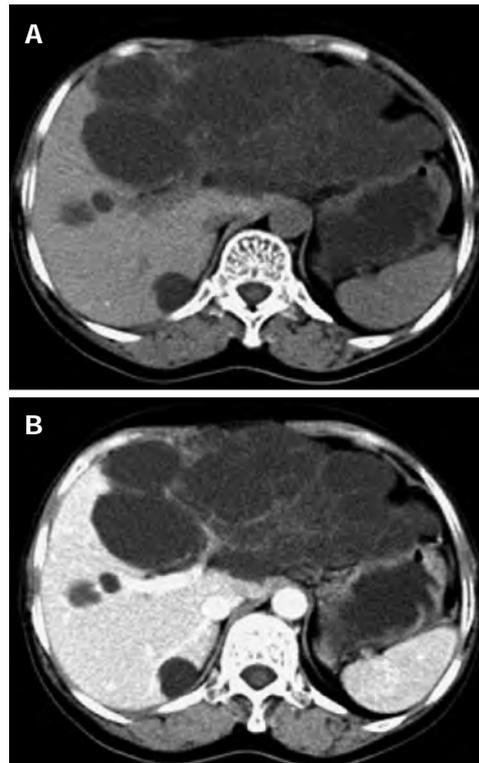


Figure 1 Transverse computed tomography scan showed a left hepatic multiloculated cystic mass measuring 15.0 cm × 9.1 cm (A) and contrast computed tomography showing enhanced septum of the tumor (B). Simultaneously, multiple sizes of hypoattenuating shadows without enhancement were seen in the right liver lobe.

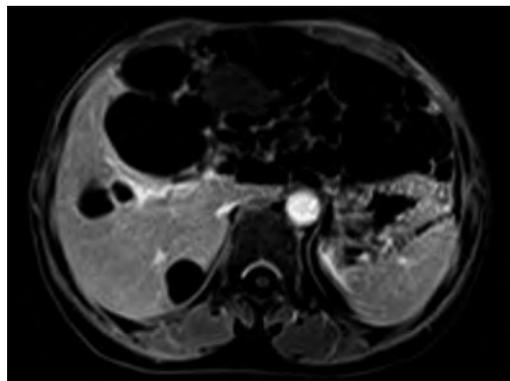
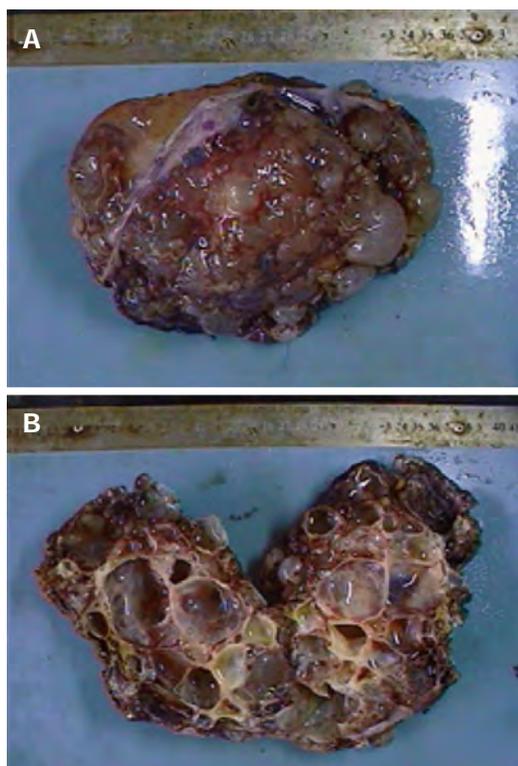


Figure 2 T1-weighted imaging revealed a large cystic tumor measuring approximately 18.0 cm × 9.0 cm originating from the left liver lobe.

antigen (CA) 19-9, CA-125 and  $\alpha$ -fetoprotein (AFP) levels were normal. On the basis of these findings, the patient was diagnosed with a hepatobiliary cystadenoma and polycystic liver.

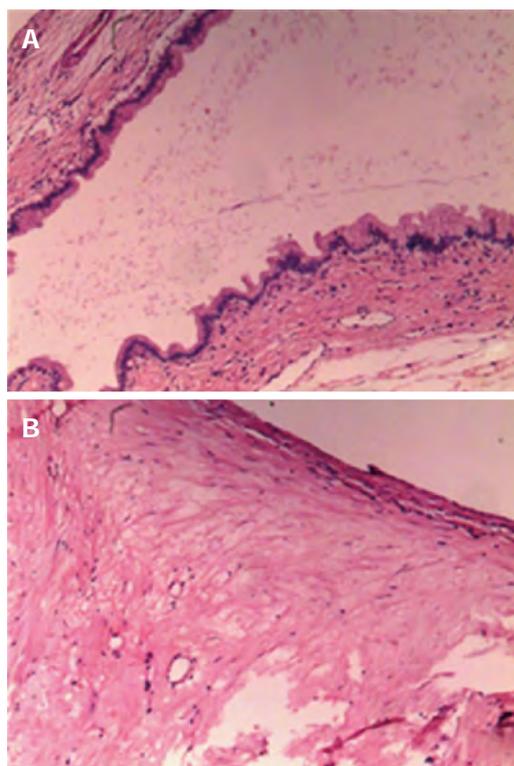
The patient underwent an exploratory laparotomy with left hepatic lobectomy, right liver cyst fenestration, and cholecystectomy through a right subcostal incision. A large cystic mass presenting with grape-like blisters was located on the surface of the left lobe of the liver. It was well encapsulated and essentially without invasion into any other structures, and it was completely excised.



**Figure 3** Resected left liver specimen showed a multilocular cystic lesion measuring 15 cm × 9 cm × 8 cm, covered with bullate nodules on the cut surface (A), and opened specimen filled with grayish yellow but clear fluid, the inner surface was smooth without any masses or excrescences (B).

On gross examination, the resected left liver specimen showed a multilocular cystic lesion measuring 15 cm × 9 cm × 8 cm, covered with bullate nodules on the cut surface (Figure 3A). The cyst contained grayish yellow but clear fluid with no connection to the bile duct, and the wall was smooth. No masses or excrescences were noted on the inner surface (Figure 3B). Microscopically, the cystic lesion was lined by a single layer of cuboidal to columnar epithelial cells (Figure 4A). The cell morphology was normal and not pleomorphic. A stroma with proliferating fibrous tissue and a small number of inflammatory cells was underlying the epithelium (Figure 4B). Typical ovarian-like stroma was absent. The histopathology was homogeneous and uniform throughout the lesion. The surgical margins were negative and a final diagnosis of hepatobiliary cystadenoma was established. It also showed chronic inflammation of the gallbladder of the excised specimen.

The patient was seen 1 mo after surgery in the clinic. She was able to eat normally and had no abdominal discomfort. She was monitored with abdominal US for recurrence after operation. The abdominal US revealed some small cysts in the right liver lobe, whereas serological tests for CEA, CA19-9, CA-125 and AFP were within normal ranges. She was scheduled for a repeat US in 3 mo.



**Figure 4** Microscopic evaluation showed a single layer of cuboidal to columnar epithelial cells (A) with underlying stroma with proliferating fibrous tissue and a small number of inflammatory cells (B) (hematoxylin and eosin stain, × 100).

## DISCUSSION

BCA is a cystic benign tumor which that originates from intrahepatic or extrahepatic biliary ducts, and it is also called hepatobiliary cystadenoma. BCA represents < 5% of cystic liver disease cases<sup>[1]</sup>. The cystadenoma is predominantly intrahepatic in origin and rarely seen in extrahepatic bile ducts or the gallbladder<sup>[2]</sup>. About 90% of BCAs are located intrahepatically, with a slight predilection for the right hepatic lobe. Its size varies from 3-4 cm to a giant cyst of 20-30 cm<sup>[3]</sup>. Here, we reported a giant intrahepatic BCA located in the left hepatic lobe in a 57-year-old woman.

The etiology of BCA is still unclear, although abnormal embryonic development resulting in ectopic foregut or gonadal epithelium sequestered in the liver has been proposed recently<sup>[4]</sup>. It seems that BCA has specific epidemiological characteristics. Wang *et al.*<sup>[5]</sup> reported that the majority of intrahepatic BCA cases occurred in women aged ≤ 60 years, which was consistent with our patient's presentation. However, 11 cases of BCA have been reported in the pediatric population<sup>[2,6,7]</sup>.

Histologically, there are three described subtypes of BCA, based on the underlying type of stroma seen beneath the cuboidal or columnar epithelium. In the first subtype, BCA, microscopically, has a dense, ovarian-like

mesenchymal stroma. This group is the most common and also occurs exclusively in women at a mean age of 40 years. The second subtype has non-ovarian-like stroma and is characterized by the absence of a mesenchymal layer. This group may instead have fibrous, hyaline, or myxoid stroma and is more frequently seen in men with a mean age of 50 years. The third subtype is a cystadenoma that also lacks mesenchymal stroma, but is lined by eosinophilic epithelial cells, which resemble hepatocytes. This group is rare, occurs only in men, and may be a semimalignant histological variant<sup>[6]</sup>. Our patient lacked a mesenchymal stroma but instead had fibrous stroma, and was classified into the second group.

It is difficult to make an accurate diagnosis of BCA before surgery, and to date no publication has established presenting symptoms that differentiate BCA from other benign or malignant hepatic cystic diseases such as biliary cystadenocarcinoma, hepatic cyst, hydatid cyst, Caroli disease, undifferentiated sarcoma, intraductal papillary mucinous tumor, and hepatocellular carcinoma. Patients may present with abdominal pain, abdominal distention, indigestion, nausea, vomiting, and jaundice, yet patients may be asymptomatic at presentation<sup>[8]</sup>. On physical examination, an abdominal mass can be identified occasionally. Laboratory results are normal in most patients with BCA, although serum liver enzyme and bilirubinemia levels may be mildly elevated occasionally. Serum AFP, CA19-9, CA-125 and CEA levels are usually within the normal range. It was recently reported that CA19-9 may be elevated in the cystic fluid and contributes to the diagnosis of BCA before surgery<sup>[9]</sup>. US, CT and MRI play an important role in the diagnosis and antidiastole of the disease. Medical imaging demonstrates intrahepatic cystic lesions with features such as mural nodules, varying wall thickness, papillary projections, and internal septations, which could help to distinguish BCA from other cystic lesions of the liver. On color Doppler US, BCA may appear as a multiloculated anechoic cystic structure with irregular shape and intact membranes. Dense and scattered echogenic dots can be seen in the echo-free area with partitions in between, and solid and papillary echo which is connected to the cystic wall can be seen on the partition wall. Abdominal CT scan may further characterize the multilocular cystic lesion with enhanced, thin internal septations and surrounding normal liver parenchyma, whereas the intraluminal content is usually hypoattenuating. Its fibrous capsule and internal septations are often visible and help distinguish the lesion from a simple cyst. Convex papillae can be seen on the septation, although they are more common in cystadenocarcinoma. MRI may improve tissue characterization because of its high contrast resolution. On MRI, BCA appears as a hypoattenuating lesion on T1WI and hyperintensity cystic fluid on T2WI, but the signal intensity may vary depending on the properties of the cyst fluid<sup>[10,11]</sup>. On T1WI, the signal intensity may increase with protein concentration. The signal intensity of serous fluid and bile is low. In rare cases, the intensity of serous cystic content can be raised

by intracystic hemorrhage and fluid-fluid level can be present. On T2WI, septations with low signal intensity are better visualized in contrast to the high signal intensity of the cystic fluid. If jaundice is present, magnetic resonance cholangiopancreatography or endoscopic retrograde cholangiopancreatography may be considered to evaluate biliary obstruction. Most commonly, displacement and extrinsic compression of the bile ducts by the tumor is seen, and rarely, communication between the cyst and biliary tree may be observed<sup>[12]</sup>. With the development of US contrast agent in recent years, contrast-enhanced ultrasonography has become increasingly useful in the diagnosis of liver lesions. BCA manifests as a non-homogeneous rich blood supply cystic masses, the solid part and the wall nodules show strong enhancement in the arterial phase, decreased enhancement in the delayed phase, and equivalent enhancement in the portal phase. Nevertheless, the wall nodules are a little more sharply enhanced in the three phases, making it difficult to differentiate from cystadenocarcinoma. It is well known that US-guided biopsy is the most commonly used method for preoperative pathological diagnosis, and cyst fluid cytology and enzymes can be detected, but carcinomatosis may occur following cyst biopsy<sup>[9]</sup>.

Although imaging is the major diagnostic method for BCA at present, surgery is still the only means of accurate diagnosis. Definitive diagnosis requires histological examination following formal resection, liver transplant, or enucleation<sup>[13]</sup>. Resection or enucleation with clear margins is the treatment of choice for suspected BCA. Malignant degeneration and recurrence are 30% and 90%, respectively, for incompletely excised lesions<sup>[14]</sup>. Therefore, patients who only undergo treatment such as aspiration or laparoscopic fenestration would have a poor prognosis. Surgical resection is still necessary when there is diagnostic uncertainty, especially when the patient was complicated with simple or multiple liver cysts. Sometimes, intraoperative frozen sectioning should be performed, in order not to miss a neoplastic condition, such as cystadenocarcinoma. It is important for surgeons to determine the scope of the operation and for patients to receive timely surgery.

Our patient was complicated with polycystic liver disease, which was initially only diagnosed as polycystic liver. This indicates that such cases should be brought to the attention of US physicians and hepatobiliary specialists. In outpatients with diagnosis of hepatic cysts, especially multiple cysts, CT or MRI should be performed when a diagnosis of BCA is suspected.

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## Epidermal growth factor receptor and metastatic colorectal cancer: Insights into target therapies

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### Panitumumab

**Core tip:** Metastatic colorectal cancer (mCRC) remains a challenge for oncologists worldwide. Despite a very aggressive disease profile, mCRC's outcomes are improving toward last decades. Target drugs, such as cetuximab and panitumumab, acquired a main role in this scenario whether phase III trials showed interesting results in overall survival and disease control. Thus, we will briefly in this paper discuss some issues and pitfalls concerning this framework.

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### Abstract

Colorectal cancer (CRC) has high incidence and mortality worldwide. In 2012, CRC was the second most prevalent cancer among males (9%) and the third among females (8%). In recent decades, standard chemotherapy protocols combining 5-fluorouracil, leucovorin, irinotecan and oxaliplatin were important for improve survival in this set of patients. Further, biological drugs throughout epidermal growth factor receptor (EGFR) pathways showed interesting results in metastatic disease (mCRC) control when in association to standard chemotherapy regimens. Cetuximab and panitumumab are two cornerstones for mCRC treatment and are both approved in Europe and United States based on previous results phase III trials. This paper will briefly summarize those anti-EGFR therapies framework in mCRC and discusses some issues in this regard.

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**Key words:** Colorectal cancer; Epidermal growth factor receptor; *KRAS* mutation; Chemotherapy; Cetuximab;

### INTRODUCTION

Colorectal cancer (CRC) has high incidence and mortality worldwide. In 2012, CRC was the second most prevalent cancer among males (9%) and the third among females (8%)<sup>[1]</sup>. The survival rates, in advanced CRC remain low; therefore, the development of new therapeutic weapons becomes a real need. Targets therapies through epidermal growth factor (EGF) and its receptor (EGFR) and also *KRAS* pathways modulation acquired a main role whether in association with standard chemotherapy<sup>[2]</sup>. Since its discovery, EGFR has been considered a good candidate for targeted cancer therapy<sup>[3,4]</sup>. It is over expressed in many types of cancers, especially CRC<sup>[5]</sup>.

### EPIDERMAL GROWTH FACTOR RECEPTOR

Although EGFR remains a controversial prognostic fac-

**Table 1** Mainly clinical trial and target therapies

Study	Design	Median PFS (mo)	Median OS (mo)	Toxicity (grade 3/4)	Genetic analyses	Response rate
PACCE trial <sup>[18]</sup>	PMAB + Bev/Ox-CT	10	19.4	Skin rash, diarrhea, infections and pulmonary embolism	KRAS status was determined in 82% tumor samples. Mutations were found in 40%	46%
	PMAB + Bev/Iri-CT + Bev/Ox-CT	11.4	24.5			48%
Peeters <i>et al</i> <sup>[22]</sup>	Panitumumab-FOLFIRI (in the WT KRAS subpopulation)	5.9	14.5 <sup>1</sup>	Toxicities associated with anti-EGFR therapy	KRAS status was available for 91% of patients: 597 (55%) with wild-type KRAS tumors, and 486 (45%) with mutant KRAS tumors	Improved to 35% vs 10% with the addition of panitumumab
	FOLFIRI (in the WT KRAS subpopulation)	3.9	12.5 <sup>1</sup>			
PRIME study <sup>[28]</sup>	Wild-type KRAS stratum Panitumumab + FOLFOX (4)	9.6	23.9 <sup>1</sup>	Toxicities associated with anti-EGFR therapy	KRAS results were available for 1100 (93%) patients	55%
	Mutant KRAS stratum Panitumumab + FOLFOX (4)	8.0	19.7 <sup>1</sup>			48%
	Panitumumab + FOLFOX (4)	7.3	15.5 <sup>1</sup>			40%
	FOLFIRI (4)	8.8	19.3 <sup>1</sup>			40%
COIN trial <sup>[29]</sup>	Ox and 5FU (arm A) in KRAS wild-type tumours	8.6 <sup>1</sup>	17.9 <sup>1</sup>	NA	565 (43%) had KRAS mutations	57%
	Ox and 5FU plus cetuximab (arm B) in KRAS wild-type tumours	8.6 <sup>1</sup>	17.0 <sup>1</sup>	Skin rash and gastrointestinal toxic effects		64%
NORDIC-VII <sup>[20]</sup>	Standard Nordic FLOX (arm A)	7.9 <sup>1</sup>	20.4 <sup>1</sup>	The regimens were well tolerated	KRAS and BRAF mutation analyses were obtained in 498 (88%) and 457 patients (81%) respectively	41%
	Cetuximab and FLOX (arm B)	8.3 <sup>1</sup>	19.7 <sup>1</sup>			49%
	Cetuximab combined with intermittent FLOX (arm C)	7.3 <sup>1</sup>	20.3 <sup>1</sup>			47%

<sup>1</sup>Without statistical significance. PFS: Progression-free survival; OS: Overall survival; PMAB: Panitumumab; Bev: Bevacizumab; Ox:CT: Oxaliplatin-based chemotherapy; Iri-CT: Irinotecan-based chemotherapy; 5FU: 5-fluorouracil; FOLFOX/FLOX: Fluorouracil, leucovorin and oxaliplatin; FOLFIRI: Fluorouracil, leucovorin and irinotecan; NA: Not applicable.

tor, this expression-stage association may play a crucial role in the decision to initiate an adjuvant treatment toward *KRAS* mutation assessment<sup>[6]</sup> as it will be discussed below.

However, not all patients have a good response to anti-EGFR monoclonal antibodies, and given the risks for adverse effects associated with their use and their substantial cost, there is particular interest in identifying predictors of treatment benefit or lack thereof<sup>[2]</sup>. Genetic alterations may explain the resistance to anti-EGFR therapies<sup>[7]</sup>. In current clinical practice, *KRAS* mutation (codon 12 and 13) is mainly responsible for primary resistance to the EGFR target drugs, particularly cetuximab and panitumumab<sup>[8]</sup>. Thus the advantages of anti-EGFR monoclonal antibody treatment of colorectal cancer may be limited to *KRAS* wild-type patients<sup>[9]</sup>.

## METASTATIC COLORECTAL CANCER

Currently, we know that many monoclonal antibodies has been approved by Food and Drugs Administration (FDA) and European Medicine Agency for the treatment of mCRC: cetuximab and panitumumab in *KRAS* wild-type patients<sup>[5,9]</sup> and bevacizumab for those harbor codon 12 or 13 mutation<sup>[10,11]</sup>. These drugs are used in association with

chemotherapy in patients with mCRC or maintenance therapies in chemorefractory tumors (Table 1). In overall, current guidelines advocate the use of the following regimens as initial standard chemotherapy for mCRC: fluorouracil, leucovorin, and oxaliplatin-based chemotherapy (FOLFOX), fluorouracil, leucovorin, and irinotecan-based chemotherapy (FOLFIRI), capecitabine plus oxaliplatin (CapeOx or XELOX)<sup>[12,13]</sup>, and fluorouracil, leucovorin, oxaliplatin and irinotecan-based chemotherapy (FOLFOXIRI)<sup>[14]</sup>. The addition of a biological agent, such as anti-vascular endothelial growth factor (bevacizumab)<sup>[15]</sup> or anti-EGFR (cetuximab or panitumumab), in *KRAS* wild-type, will depends on patients *KRAS* profile, fitness and related- clinical co-morbidities.

However, we should be aware for the toxicity profile. Most common anti-EGFR adverse events<sup>[16]</sup> are the skin acneiform rash, xeroderma, hypomagnesemia, diarrhea and nausea<sup>[17]</sup>. Hecht *et al*<sup>[18]</sup> assessed panitumumab plus bevacizumab versus regular chemotherapy (oxaliplatin and irinotecan-based) as first line treatment for mCRC and showed no outcomes benefit, but only increase in toxicity profile, particularly diarrhea, infections and pulmonary embolism<sup>[19]</sup>. The increased in the toxicity can be explicated by dual-pathway inhibition in combination with chemotherapy<sup>[18]</sup>. In this study the patients were

enrolled onto one of two cohorts per investigator arm: a fluorouracil, leucovorin, and oxaliplatin-based chemotherapy (FOLFOX) cohort or a fluorouracil, leucovorin, and irinotecan-based chemotherapy (FOLFIRI) cohort, each with bevacizumab. Anyway, panitumumab given with FOLFOX or with FOLFIRI in the absence of bevacizumab appears to be well tolerated in other studies.

Tveit *et al.*<sup>[20]</sup> evaluated in mCRC patients the efficacy of cetuximab plus bolus fluorouracil/folinic acid and oxaliplatin, administered continuously or intermittently as first line regimen. This study did not show significant benefit compared with the FOLFOX regime. For another hand in third-line treatment of patients with chemotherapy-refractory mCRC, cetuximab provides a substantial prolongation of progression-free-survival (PFS) and overall survival<sup>[21]</sup>. Similarly, panitumumab plus FOLFIRI has shown significantly improved in PFS and was well-tolerated as second-line treatment in patients with wild-type *KRAS* mCRC<sup>[22]</sup>.

Plus, the combination of cetuximab plus FOLFIRI as first-line chemotherapy in wild-type *KRAS* tumors also can reduce the risk of progression of mCRC as compared with FOLFIRI alone<sup>[23]</sup>. Moreover, we should note that the toxicity of FOLFIRI plus cetuximab combination was superior to FOLFIRI regimen alone (notably skin reactions). Notwithstanding, patients with *KRAS* wild-type locally advanced rectal cancer with the addition of cetuximab to chemoradiation regimen based on irinotecan plus capecitabine showed no benefit compared to the use of chemoradiation alone<sup>[24]</sup>. Further, in spite of we have focused our attention to *KRAS* mutations; there are others biomarkers that are also implicated in colorectal cancer outcomes, such as *BRAF* mutation. *BRAF*-mutant tumors have worse prognosis<sup>[25]</sup>. Recently *BRAF* inhibitor, vemurafenib, was approved by the FDA for treatment of patients with *BRAF*-mutant metastatic melanoma<sup>[26]</sup>. It is expected that in the near future other *BRAF* inhibitors are developed and maybe directed to mCRC.

## CONCLUSION

In summary, the most recent studies aim to demonstrate not only the efficacy and safety of the target molecules discussed above, in particular cetuximab and panitumumab, but also how these new agents act in conjunction with conventional chemotherapy. Currently, mCRC molecular profile assessments acquired a main role for oncologists worldwide due to the possibility of personalizing treatments approaches for mCRC patients and thus improve survival outcomes as well as quality of life<sup>[27-29]</sup>. In addition, the choice to use bevacizumab, cetuximab or panitumumab in association with standard chemotherapy (FOLFOX or FOLFIRI) for mCRC framework run toward patients fitness, acceptable toxicities profiles, survival outcome and mainly pharmaco-economic evaluation of those drugs in this setting.

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## Pain in chronic pancreatitis: Managing beyond the pancreatic duct

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### Abstract

Chronic pancreatitis (CP) continues to be a clinical challenge. Persistent or recurrent abdominal pain is the most compelling symptom that drives patients to seek medical care. Unfortunately, in spite of using several treatment approaches in the clinical setting, there is no single specific treatment modality that can be earmarked as a cure for this disease. Traditionally, ductal hypertension has been associated with causation of pain in CP; and patients are often subjected to endotherapy and surgery with a goal to decompress the pancreatic duct. Recent studies on humans (clinical and laboratory based) and experimental models have put forward several mechanisms, including neuroimmune alterations, which could be responsible for pain. This might explain the partial or no response to single modality treatment in a significant proportion of patients. The current review discusses the recent concepts of pain generation in CP and evidence based therapeutic approaches (other than ductal decompression) to handle persistent or recurrent pain. We focus primarily on parenchymal and neural components; and discuss the role of antioxidants

and the existing controversies, drugs that interfere with neural transmission, pancreatic enzyme supplementation, celiac neurolysis, and pancreatic resection procedures. The review concludes with the treatment approach that we follow at our institute.

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**Key words:** Pain; Chronic pancreatitis; Nociception; Neuroplasticity; Antioxidant micronutrients; Pregabalin; Pancreatic enzymes

**Core tip:** Pain in chronic pancreatitis (CP) has multiple but simultaneously occurring mechanisms. Recent data have shown expression of nociceptors and neurotrophic factors in different neural locations. The expression of these and other neural chemokines (fractalkine) have positive correlation with pain. Pain also results from global sensitization. Among the therapeutic modalities, beneficial effects have been demonstrated with methionine containing antioxidant micronutrients supplements and pregabalin. Of the pancreatic enzymes, only non-enteric coated preparations might benefit a subgroup of patients. The threshold for performing celiac neurolysis should be high in view of variable response across clinical trials.

Talukdar R, Reddy DN. Pain in chronic pancreatitis: Managing beyond the pancreatic duct. *World J Gastroenterol* 2013; 19(38): 6319-6328 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6319.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6319>

### INTRODUCTION

Pain in chronic pancreatitis (CP) is as enigmatic as the disease itself. There is currently no definitive cure for the illness; and treatment usually centers on pain relief and

management of exocrine and endocrine dysfunction. 85%-90% patient will have abdominal pain at presentation<sup>[1]</sup>; and in our experience over two-third of patients would present with ductal calculi and/or stricture (unpublished data). Traditionally, pain in CP has been largely associated with ductal hypertension. However, as evident from the literature, recurrence of pain is common even after ductal decompression in the form of extracorporeal shock wave lithotripsy (ESWL), endoscopic retrograde cholangiopancreatography (ERCP) or lateral pancreaticojejunostomy. Over the past several years, a host of pain mechanisms have been proposed and demonstrated directly or indirectly in humans<sup>[2]</sup>. Most important among these are oxidative stress and inflammation induced pancreatic nociception, pancreatic neuropathy/neuroplasticity and central neuroplasticity. There appears to be significant cross talk among the different mechanisms, which could explain the partial or no success of single modality treatment approaches. This mandates a holistic and conceptualized approach to pain management in CP, aided by the little evidence available.

This review addresses the current concepts of genesis of pain in CP and evidence based management approaches focusing primarily on the parenchymal and neural components.

## PAIN MECHANISMS

### Nociception

Nociception refers to the perception of pain sensation as a result of activation of pain receptors (nociceptors). The proteinase-activated receptor 2 (PAR-2) and the transient receptor potential vanilloid 1 receptors are two discrete types of nociceptors that have been shown to be present in the pancreas specific sensory nerves and dorsal root ganglia<sup>[3,4]</sup>. It is now known from experimental models that even sub-inflammatory doses of trypsin could bind to the PAR-2 receptor, thereby suggesting trypsin as a potential nociceptive stimulus, independent of its inflammatory role<sup>[4]</sup>. Other nociceptive stimuli that have been proven or speculated to stimulate pancreatic nociceptors include trypsin, alcohol metabolites, protons, bradykinin, hydrogen sulphide, serotonin and calcium<sup>[5]</sup>. The primary sources of intrapancreatic trypsin are the mast cells that infiltrate the pancreatic nerves in CP. The latter mediators are known to be released by injured acinar cells. Recently, another nociceptor namely the ligand-gated cation channel Transient receptor potential ankyrin 1 has also been shown to cause pancreatic inflammation and visceral hypersensitivity<sup>[6]</sup>.

Other than the above-mentioned ligands, several neurotrophic factors like nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial-derived neurotrophic factor and artemin are expressed locally in the pancreas in response to inflammation and bind to specific receptors at different regions within the nerves (Figure 1)<sup>[7-10]</sup>. These factors, after binding to the respective receptors cause nociceptive sensitization and neural

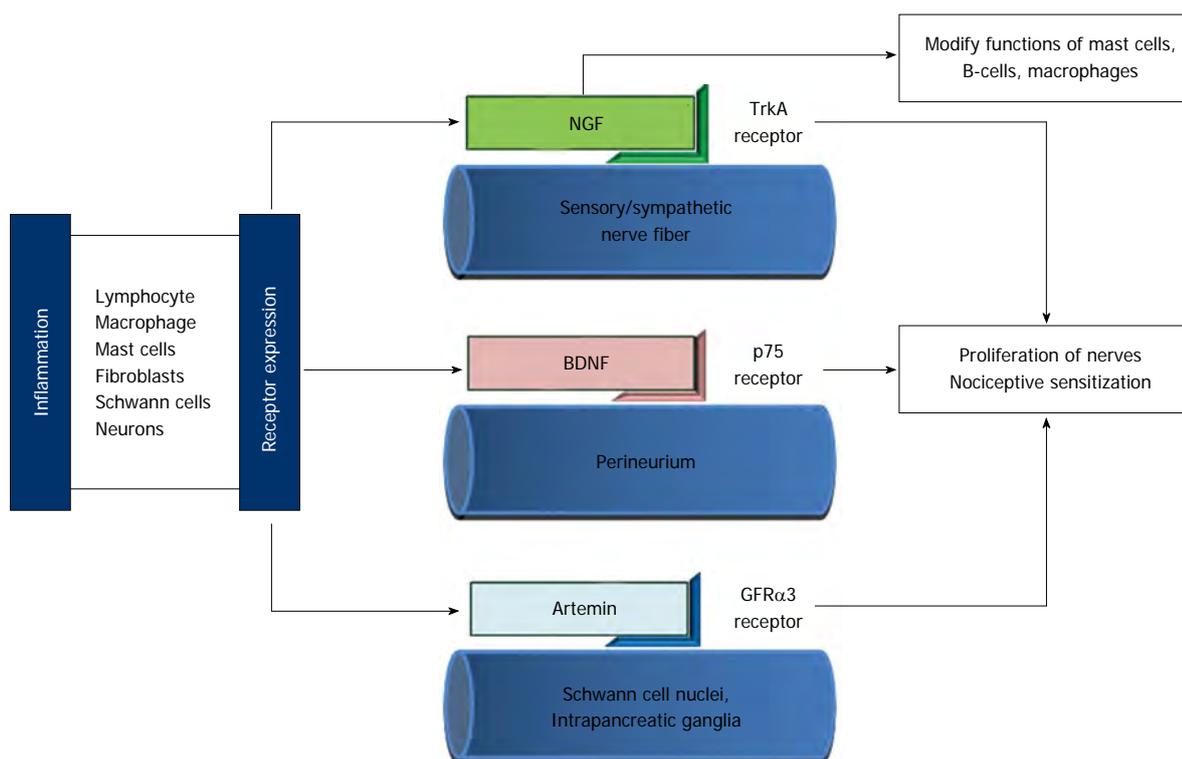
proliferation. Interestingly, the expression of TrkA, BDNF and artemin has been found to correlate with the severity of pain in patients with CP<sup>[2]</sup>.

Even though ductal hypertension had been considered to be a major cause of pain in CP, the mechanism was not known clearly. Recently, it was shown in experimental models that increase in pressure can activate pancreatic stellate cells (PSC), which in turn can generate oxidative stress<sup>[11]</sup>. Furthermore, ethanol and smoking can by itself lead to oxidative stress within the PSCs<sup>[12,13]</sup>. Oxidative stress is capable of generating a pro-inflammatory state by means of activating immune cells, increasing expression of pro-inflammatory cytokines, and activating cytokine receptors and transcription factors (*e.g.*, tumor necrosis factor alpha, NF- $\kappa$ B)<sup>[2]</sup>. This could indirectly or directly sensitize the intrapancreatic pain receptors. The response of pain in CP along with normalization of circulating oxidant stress markers after treatment with high-dose anti-oxidants is a testimony to this.

Once activated, the pain receptors generate an action potential in the first order sensory neurons of spinal levels T5 to T9. The action potential travel forward (antegrade) to the dorsal horn (gray matter) of the spinal cord where it results in the release of the neurotransmitters glutamate, calcitonin gene related peptide (CGRP) and substance P<sup>[14]</sup>, which subsequently excites the second order neurons in the dorsal horn *via* N-methyl D-aspartate,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and the neurokinin 1 (NK-1) receptors<sup>[15]</sup>. The nociceptive transmission is then propagated through the ascending pathways in the spinal cord white matter to the thalamus, from where third order neurons relay to the sensory cortex, limbic system and the thalamus for cognitive and affective integration of pain. The sympathetic efferent cell body is the other sensory component to which the first order pancreatic nociceptive neurons project. This in turn relays to the celiac plexus *via* the greater splanchnic nerves and finally synapses with the second order sympathetic neurons. Axons of these sympathetic neurons then travel cephalad in the vagal trunks<sup>[2]</sup>.

### Neural mechanisms

Several neuroimmune and neuroplastic mechanisms have been described in CP pain over the recent years in humans and experimental models. The most conspicuous neural changes in the intrapancreatic nerves include: (1) infiltration of inflammatory cells (especially mast cells and eosinophils)<sup>[16]</sup>; (2) neural edema and perineural disruption<sup>[17]</sup>; (3) Schwann cell (glial cell in peripheral nerves) proliferation<sup>[18]</sup>; and (4) neural hypertrophy and sprouting<sup>[19]</sup>, to name a few. The magnitude of these changes has been shown to correlate with the severity of pain, thereby ascribing them a causal role for neuropathic pain in CP. Possible factors that could be responsible for neural inflammation in the pancreatic nerves include glutamine, CGRP, substance P, and fractalkine. Some of these mediators can travel retrograde (antidromic) from the dorsal horn to the intrapancreatic nerve end-



**Figure 1** Schematic diagram representing neuroimmune mechanisms of pain in patients with chronic pancreatitis. The TrkA receptors (for nerve growth factor, NGF) are expressed on the sensory and sympathetic nerve fibres, p75 (for brain derived neurotropic factor, BDNF) on the perineurium and glial cell line-derived neurotrophic factor receptor  $\alpha 3$  (GFR $\alpha 3$ ) (for Artemin) on the Schwann cell nuclei and intrapancreatic ganglia. The receptor expression is mediated by inflammation involving inflammatory cells and neural elements.

ing and induce chemotaxis of inflammatory cells<sup>[2,20,21]</sup>. Furthermore, overexpression of two important markers, namely, nestin and growth-associated protein-43 has been demonstrated in pancreas of human CP<sup>[19]</sup>. These two are markers of neuroplasticity/neural regeneration and are responsible for Schwann cell and neural growth. The composite of these findings and the associated pain clearly points towards profound neural remodeling within the pancreas (pancreatic neuroplasticity). These changes have important bearing on pain processing in central nervous system both at the level of the spinal cord and higher centers. Continuous sensitization of the intrapancreatic nociceptors due to persistent inflammation results in continuous stimulation of second order neurons present the dorsal horn of the spinal cord, a phenomenon called global sensitization<sup>[22]</sup>. The clinical surrogate of global sensitization is an increase in the area of referred pain due to convergence of afferent fibres from different visceral and somatic organs on the same hyperexcitable secondary spinal neurons. This has been demonstrated recently in patients with CP, in whom the areas involved in referred pain in response to esophageal, gastric and duodenal stimulation were significantly higher than those in controls<sup>[23]</sup>. Global sensitization results in two important phenomenon, mechanical allodynia (generation of pain after a physiological or non-noxious stimulus) and inflammatory hyperalgesia (amplified pain response to normal or minimal pain stimuli). The other manifestation of global sensitization is an increasingly painful response

to repetitive but isointense stimuli. This is known as temporal summation, which has been clearly demonstrated in patients with CP<sup>[24,25]</sup>. It has also been demonstrated that early event-related brain potentials are altered in several key pain processing areas in the cerebral cortex in response to visceral stimulation in patients with CP<sup>[23]</sup>. This, along with a posteromedial shift in the electrical dipoles indicates significant neural reorganization in the cerebral cortical pain processing architecture. This is central neuroplasticity. Other evidence of central neuroplasticity in CP has come from clinical studies that have shown increase in theta activity on electroencephalogram (EEG) and increased activity in the right secondary somatosensory area on magnetic resonance spectroscopy<sup>[26,27]</sup>. Furthermore, abnormalities have been demonstrated in the descending inhibitory pathways from the cortex, which tilts the balance between these and ascending excitatory pathways in favour of more central pain processing abnormalities<sup>[28]</sup>.

Figure 2 depicts a conceptual model of pain in patients with CP.

## CLINICAL EVALUATION

Even though different mechanisms for pain in CP have been proposed and demonstrated, there are currently no easily available and clinically validated tools to identify the type of pain. A clinical history of new or wider areas of referred pain could be an indicator of the develop-

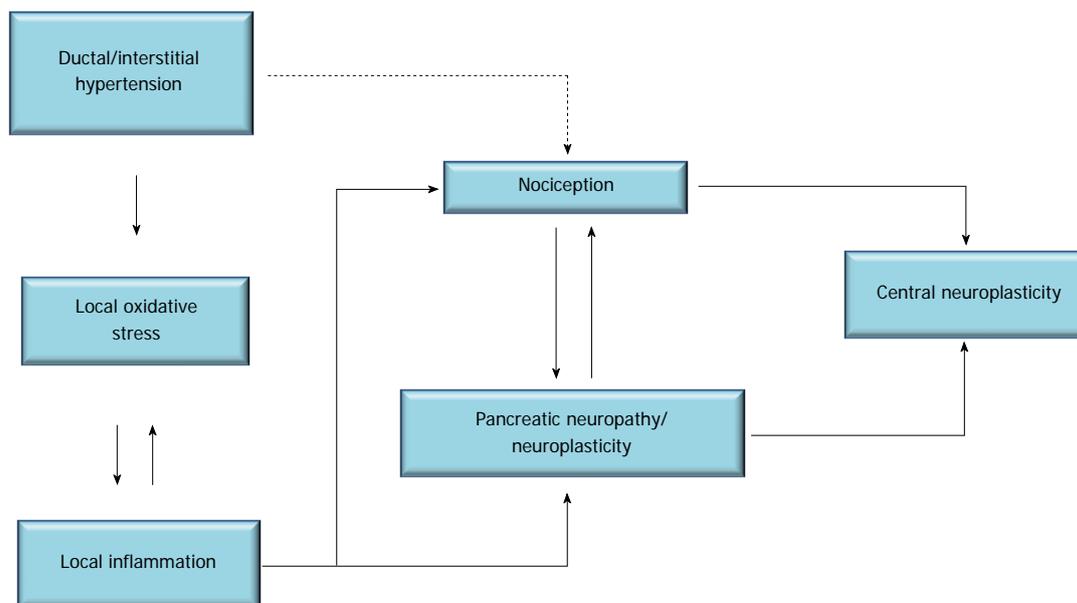


Figure 2 Schematic representation of the conceptual framework of pain mechanisms in chronic pancreatitis.

ment of neuropathy. An objective questionnaire based tool namely painDETECT has been used to evaluate neuropathic pain in the context of radiculopathy<sup>[29]</sup>. This questionnaire evaluates components pertaining to neuropathic pain (for *e.g.*, burning/tingling nature of pain); and could have a potential use in patients with CP to assess the neuropathic component of the total pain. Persistence of theta wave on EEG is another proven feature of development of central neuroplasticity<sup>[26]</sup>. However, this has not been tested and validated in large multicenter studies to be recommended for use in the routine clinical setting. Furthermore, even though few groups have used quantitative sensory testing for evaluation of pain in CP, this may not be feasible widely<sup>[30]</sup>.

## PAIN MANAGEMENT BEYOND DUCTAL DECOMPRESSION

In routine pancreatology practice, usually three broad categories of CP patients with recurrent or persistent pain are encountered, namely those with ductal obstruction (with calculi or stricture), those after ductal decompression (post-endothrapy/surgery) with a dilated duct and those with a non-dilated duct but only parenchymal changes. In the first category, ductal decompression in the form of endothrapy (ESWL with/without ERCP and ductal stenting) is the current standard of care<sup>[31,32]</sup>. Discussion of management of this group of patients is out of the scope of the current review. In the second category, it is important to rule out recurrent stones (which may be radiolucent), stricture, local complications (like inflammatory mass, biliary obstruction, pseudocysts), and cancer. In the absence of these, recurrent pain in this group of patients (and also in the third group) is most likely to be associated with predominant neural mechanisms resulting from interstitial hypertension, tissue

ischaemia, and neural inflammation. For the management of chronic and recurrent pain in CP, following treatment modalities have been practiced.

### Analgesics

For short-term relief of pain in CP, the World Health Organization pain ladder, starting with an nonsteroidal anti-inflammatory drugs may be followed<sup>[33]</sup>. It is a common practice in many western countries to use opiates long-term to ameliorate chronic and recurrent pain. However, even though high potency opiates like morphine and analogues are effective, they should be avoided as a first line drug as far as possible due to the risk of drug dependence and potentiation of side effects, including narcotic bowel syndrome. Furthermore, morphine and codeine can cause activation and degranulation of mast cells, thereby overriding the beneficial effect while worsening the inflammation and pain<sup>[34]</sup>. Tramadol, though a low potency selective  $\mu$ -opiate receptor agonist, has been shown to be as effective as higher potency narcotics but with a significantly better safety profile<sup>[35]</sup>. Other effective alternatives for severe continuing pain include epidural buprenorphine and transdermal fentanyl (patch)<sup>[36]</sup>.

### Antioxidant micronutrients

The primary aim of antioxidant micronutrient therapy in CP is to supply methyl and thiol moieties for the transsulfuration pathway, which is essential for protection against reactive oxygen species (ROS) mediated electrophilic stress<sup>[37]</sup>. It has been demonstrated in clinical studies that there is a significant reduction in antioxidant defense in patients with CP. Studies from the United Kingdom, India and Spain have used a antioxidant cocktail consisting of methionine, organic selenium, ascorbic acid,  $\beta$ -carotene, and  $\alpha$ -tocopherol; out of which 2-4 g/d of methionine (which preserves the transsulfuration path-

**Table 1** Studies evaluating the role of antioxidant micronutrients on clinical outcomes, including pain, in patients with chronic pancreatitis

Ref.	Study type	Antioxidant micronutrients used	Indications; study duration	Outcomes
Uden <i>et al</i> <sup>[38]</sup> 1990	DB double dummy cross over	Vit C; Vit E; Beta carotene; Selenium; Methionine	<i>n</i> = 20 (ACP, ICP, IAP) 20 wk	↓ in VAS
De las Heras Castaño <i>et al</i> <sup>[39]</sup> 2000	Open label	Vit C; Vit E; Beta carotene; Selenium; Methionine	<i>n</i> = 19 (ACP, ICP, RAP) 12 mo	↓ in VAS, ↓ admission, ↑ exocrine fn
Dite <i>et al</i> <sup>[40]</sup> 2003	Open label	Vit C; Vit E	<i>n</i> = 70 (ACP, ICP) 12 mo	Pain abolished in 44%
Kirk <i>et al</i> <sup>[41]</sup> 2006	DB RCT cross over	Vit C; Vit E; Beta carotene; Selenium; Methionine	<i>n</i> = 19 (all ACP) 20 wk	↑ QOL
Bhardwaj <i>et al</i> <sup>[42]</sup> 2009	DB RCT	Vit C; Vit E; Beta carotene; Selenium; Methionine	<i>n</i> = 127 (ACP, ICP); 80% power; 6 mo	32% patients pain free ↓ No. of painful days ↓ analgesic need
Siriwardena <i>et al</i> <sup>[43]</sup> 2012	DB RCT	Vit C; Vit E; Beta carotene; Selenium; Methionine	<i>n</i> = 70 (ACP, ICP); 80% power; 6 mo	↔ pain ↔ QOL

↓: Indicates reduction; ↑: Indicates improvement; ↔: Indicates equivocal. DB: Double blind; RCT: Randomized controlled trial; QOL: Quality of life; Vit: Vitamin; ACP: Alcoholic chronic pancreatitis; IAP: Idiopathic acute pancreatitis; RAP: Recurrent acute pancreatitis; fn.: Function; ICP: Idiopathic chronic pancreatitis.

way in acinar cells) is believed to be the most important. Table 1 shows the clinical trials that have evaluated the effect of antioxidant micronutrient supplementation on pain relief in CP<sup>[38-43]</sup>. The largest randomized controlled trial (from India) with 127 patients that used organic selenium (600 µg), ascorbic acid (0.54 g), β-carotene (6000 IU), α-tocopherol (270 IU) and methionine (2 g) for six months demonstrated a significant reduction in the number of painful days compared to placebo ( $7.4 \pm 6.8$  d *vs*  $3.2 \pm 4.0$  d, respectively;  $P < 0.001$ ) and use of analgesic tablets per month<sup>[42]</sup>. There was also significant concomitant reduction in markers of oxidative/electrophilic stress like TBARS and improvement in antioxidant capacity. However, the clinical efficacy found in this trial was negated by the most recent randomized trial from Manchester (ANTICIPATE study), which concluded that even though micronutrients increased the antioxidant levels in blood, they did not produce adequate pain relief<sup>[43]</sup>. It is important to note that in contrary to the Indian study, patients in the ANTICIPATE study were on high dose of narcotics, many continued to consume alcohol and many did not respond to other forms of therapy either<sup>[44,45]</sup>. We believe that the optimal dose of antioxidant micronutrients confers definite benefit in terms of pain relief in carefully selected patients with CP. Even though it is advisable to monitor plasma glutathione and micronutrient concentrations, and titrate doses accordingly, in practice this may not be feasible.

In addition to the fixed dose antioxidant cocktail regimen, it is also important to give dietary advise on intake of anti-oxidant rich diet, and avoid practices that can adversely affect the bioavailability of dietary antioxidants (*e.g.*, vegetables cooked in high temperature)<sup>[46]</sup>. Folate deficiency can hinder with methionine recovery for the transsulfuration pathway<sup>[2,47]</sup>. Therefore folic acid supplementation could provide additional benefits especially to the alcoholic CP patients.

#### Drugs that interfere with neural transmission

In clinical practice, tricyclic antidepressants (like amitryp-

tiline) and serotonin-norepinephrine reuptake inhibitors (like duloxetine) are frequently used for refractory pain in CP, based on the observed benefits in other neuropathic states. Similarly, anticonvulsants like gabapentin and pregabalin, which are first line drugs for diabetic neuropathy have also been used. However, among all these agents, only pregabalin have been tested in a randomized controlled setting. A recent randomized controlled trial (RCT) evaluated the effect of increasing doses (150-600 mg/d) of pregabalin for three weeks on pain in 64 patients with CP; and demonstrated that there was significant reduction in pain score in the pregabalin arm [ $-36\%$  (95%CI:  $-43 - -29$ )] *vs*  $-24\%$  (95%CI:  $-31 - -16$ );  $P = 0.02$ ]<sup>[48]</sup>. Significant improvement was also observed in the patient's global impression of change at the end of the study. Ninety-one percent of patients had some adverse events in this trial, of which the most common were neurological (feeling drunk in 35%, and light-headedness in 12%). Rest of the adverse events was similar to those in the placebo arm. The number (proportion) of patients with serious adverse events in the pregabalin and placebo patients was 4 (12%) and 2 (7%) respectively; and this difference was not statistically significant. Pregabalin is α2δ receptor blocker that decreases presynaptic release of glutamate, noradrenaline and substance P; and has been shown to improve pain in CP by inhibiting central sensitization<sup>[49]</sup>. Studies have shown that the inhibition of central sensitization is manifested by normalization of the theta band on EEG, particularly in the parietal lobe<sup>[50]</sup>. These studies does give a comprehensive mechanistic insight of the beneficial role of pregabalin on chronic pain in CP. Further long-term follow-up studies would complement the current evidence and provide data on the long term efficacy of the drug.

#### Pancreatic enzymes

Pancreatic enzymes have been often used to control pain in CP. This is based on the hypothesis that proteases in the enzyme supplement would inhibit overstimulation of duodenal cholecystokinin (CCK) receptors, which will

in turn inhibit the food induced feedback loop thereby putting the pancreas to rest. However, meta-analysis of six double-blinded RCTs from 1983-1995 involving 186 patients concluded that pancreatic enzymes confer no benefit in pain relief<sup>[51]</sup>. Similarly, a Cochrane Systematic Review of 10 RCTs (2 parallel design and 8 cross over) involving 361 patients found equivocal pain relief, fecal fat excretion and improvement in quality of life in the pooled analysis<sup>[52]</sup>. However, the individual trials in the two studies that used non-enteric coated preparations did show significant improvement in pain<sup>[53,54]</sup>, thereby fitting into the notion of reducing post-prandial pancreatic secretion by CCK receptor inhibition. This does not happen with the enteric-coated preparations because the release of these enzyme preparations (which should happen at a pH of 5.5) in the duodenum is erratic and non-uniform due to reduced ductal bicarbonate secretion in CP. The enzymes from the enteric-coated preparations are generally released more distally in the jejunum and ileum. Unfortunately, almost all currently available enzyme preparations are enteric coated and should not be prescribed for pain relief as a sole indication. Non-enteric coated enzyme preparations (wherever available) can be of some benefit to a subgroup of patients with post-prandial pain. It is important to prescribe non-enteric preparations along with a proton pump inhibitor or H<sub>2</sub> receptor blocker in order to prevent gastric acid mediated degradation of the enzymes in the stomach.

### **Celiac plexus block**

Celiac plexus block with a local anesthetic (bupivacaine) with or without a combination of steroid (triamcinolone) is another modality for treatment for pain in chronic pancreatitis. Even though this can also be performed percutaneously, endoscopic ultrasound (EUS) based procedure has better results and negligible risk of developing paraplegia, which is associated with the percutaneous technique<sup>[55]</sup>. However, the overall benefits of celiac plexus block are about 55% after 4-8 wk and a dismal 26% and 10% after 12 and 24 wk respectively<sup>[56]</sup>. Therefore, this modality should be kept as rescue therapy for patients who do not respond to conventional medical and endoscopic therapy and are not ideal surgical candidates. EUS guided celiac ganglion neurolysis with absolute alcohol is another option, but is too extreme for a benign disease, especially in the presence of additional central mechanisms of chronic pain<sup>[57]</sup>.

Side-effects of celiac block are seen in 10%-33% of patients. The most common side effects include transient self-limiting diarrhea and orthostatic hypotension, owing to the sympathetic blockade with relatively unopposed visceral parasympathetic activity<sup>[56,58]</sup>. Diarrhea usually settles in 48 h. Occasionally the patient may complain of an increase in the pain. Serious complications like retroperitoneal bleeding and peripancreatic abscess have infrequently been reported. An additional problem with the use of alcohol is the development of dense desmoplasia, which might make future pancreatic surgery difficult.

### **Surgery**

Other than drainage procedures, surgical interventions for pain control in CP includes resectional procedures like classical (Kausch Whipple) or pylorus preserving (Traverso-Longmire) pancreaticoduodenectomy, distal pancreatectomy and total pancreatectomy. Pancreaticoduodenectomy is particularly useful in pain with an associated inflammatory head mass. Even though long-term pain relief has been demonstrated in 75%-95% of patients, this procedure is associated with worsening of exocrine and endocrine functions<sup>[59,60]</sup>. Similarly, endocrine and exocrine insufficiency is seen in 80%-95% patients undergoing distal pancreatectomy<sup>[61]</sup>; and is therefore currently performed only for recurrent pain with localized disease (such as a stricture in the upstream duct not amenable to endotherapy). Total pancreatectomy is also infrequently performed in view of the associated significant morbidity. However, with the development of islet transplantation, there has been a renewed interest in select centers in total pancreatectomy with autoislet transplantation in patients with end stage CP. However, it should be borne in mind that even after removing the entire pancreas, as high as 40% of patients could still require analgesics even after 2 years of follow-up<sup>[62]</sup>. Many a time, a resectional procedure is combined with a drainage procedure, like Frey's, Beger's, Berne's and the V-shaped procedure, in order to provide the benefit of both ductal decompression and removal of a part of the inflamed pancreas (especially an inflammatory mass).

Bilateral thoracic splanchnicectomy is yet another infrequently used surgical modality that could ameliorate chronic pain in patients with CP; and have recently been shown to inhibit pain by predominantly impairing adrenomedullary function<sup>[63]</sup>.

### **Miscellaneous**

Both short and long acting octreotide have been attempted in pain management in advanced CP in small-scale studies<sup>[64,65]</sup>. Even though satisfactory pain relief has been documented, the results need to be verified in larger trials. Furthermore, whether the pain relief is better in patients with or without ductal obstruction also needs to be examined. Other than octreotide, secretin infusion has also been evaluated in a recent phase II trial with equivocal results<sup>[66]</sup>. Few of the modalities that have been used as adjuncts to medical/surgical therapy include spinal cord stimulation<sup>[67]</sup>, cognitive-behavioral therapy, and other alternative approaches for chronic pain states. However, none of these are backed by sufficient good-quality evidence to be currently recommended specifically for pain in CP.

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## **HURDLES IN MANAGING PAIN IN CP**

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Even though much have been understood on pain mechanisms in CP, there are still several hurdles in pain management in clinical practice. Firstly, several mechanisms might be simultaneously operating at any particular time

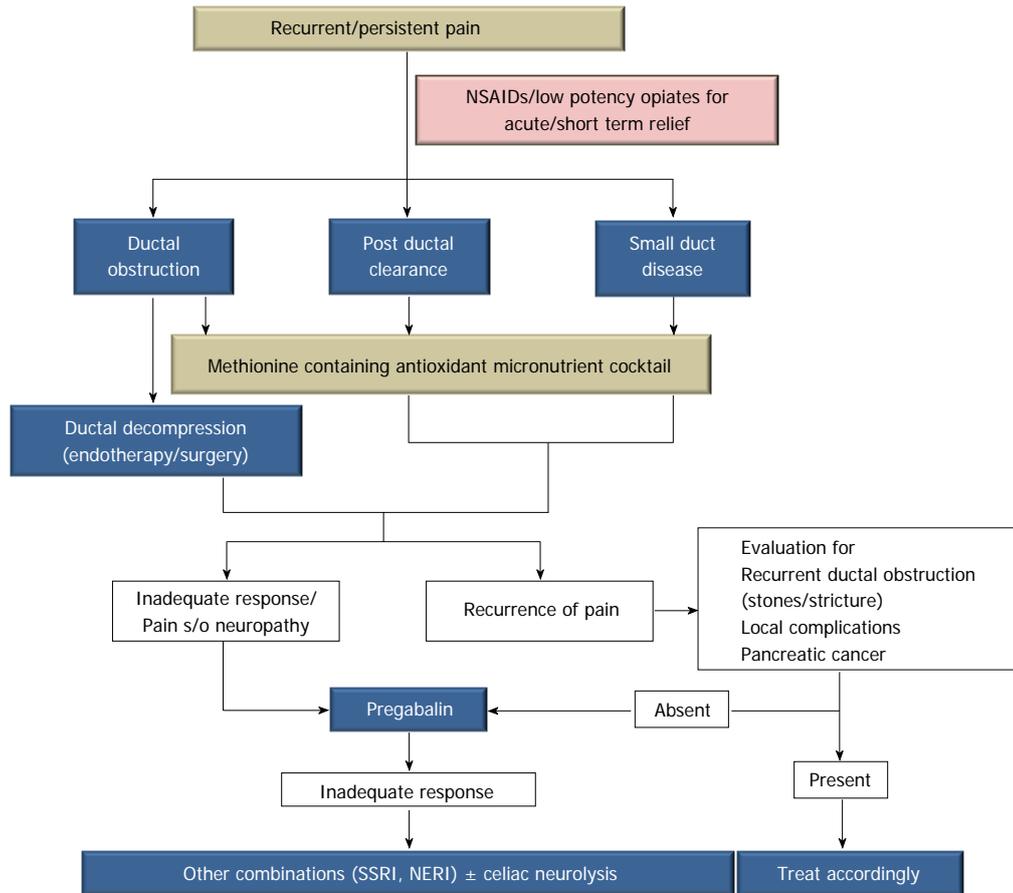


Figure 3 Management approach for recurrent and/or persistent pain in patients with chronic pancreatitis at the Asian Institute of Gastroenterology. SSRIs: Selective serotonin reuptake inhibitors; NERI: Norepinephrine reuptake inhibitor; NSAIDs: Nonsteroidal anti-inflammatory drugs.

point in a particular patient, thereby posing a question on selecting the most appropriate and optimal modality. Secondly, there are no validated objective tools that can identify the pain type, thereby precluding a fixed treatment regimen. Thirdly, there are no data to suggest an optimal duration of treatment with antioxidant and/or pregabalin in order to achieve long-term pain relief; as a result of which patients might run the risk of undertreatment or of building up excessive antioxidant micronutrients in circulation, which could impede with the physiological roles of ROS. Finally, there are no data on the efficacy or adversity of combination therapy for refractory pain.

### APPROACH TO PAIN MANAGEMENT IN CP AT ASIAN INSTITUTE OF GASTROENTEROLOGY

Figure 3 shows the treatment approach that is followed at Asian Institute of Gastroenterology. This is a composite of clinical evidence; concepts build on experimental data; and clinical experience. Methionine containing antioxidants micronutrient cocktail is started early on after the diagnosis of CP. Dose and duration of treatment is titrated according to clinical response and patient's tolerance to treatment. Patient with recurrent pain with

ductal obstruction are subjected to ductal clearance by endotherapy (ESWL with or without ERCP) or surgery (in select patients). Pregabalin is added to the regimen for patients who do not show satisfactory response to antioxidant micronutrient therapy and ductal decompression; and in those who shows clinical signs suggestive of neuropathy. In patients who have recurrence of intractable pain are evaluated for recurrence of ductal obstruction, development of local complications or cancer; and treated with additional pregabalin in the absence of these. Patients who respond sub-optimally to these regimens are treated additionally with combination of SSRI and NERI like duloxetine with or without celiac plexus block. It is important to counsel the patients thoroughly on diet and lifestyle changes like quitting alcohol and smoking all along the treatment sessions.

### CONCLUSION

Pain in CP is complex, and several independent and interdependent mechanisms may manifest simultaneously in a patient. Therefore, pain management in CP should be individualized for each patient rather than follow a fixed regimen. Recent laboratory data from human and experimental CP have opened up avenues to explore new and target specific antagonists against TRPV1, NGF, PAR2,

NK-1, CGRP and substance P.

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## Liver elastography, comments on EFSUMB elastography guidelines 2013

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### Abstract

Recently the European Federation of Societies for Ultrasound in Medicine and Biology Guidelines and Recommendations have been published assessing the clinical use of ultrasound elastography. The document is intended to form a reference and to guide clinical users in a practical way. They give practical advice for the use and interpretation. Liver disease forms the largest section, reflecting published experience to date including evidence from meta-analyses with shear wave and strain elastography. In this review comments and illustrations on the guidelines are given.

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**Key words:** Liver; Ultrasound; Elastography; Guidelines; Fibrosis

**Core tip:** The presented paper is intended to comment the "European Federation of Societies for Ultrasound in Medicine and Biology Guidelines and Recommendations on the Clinical Use of Ultrasound Elastography" and discuss the multivariate factors that have an influence on liver stiffness, and the current techniques of ultrasound elastography as well as magnetic resonance elastography.

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### INTRODUCTION

Non-invasive methods for liver stiffness (LS) assessment have been researched over decades, often mirroring the development of new drugs in the treatment of chronic liver disease. So far, two main forms of elastography have become established in clinical practice. The first is known as quasi-static or strain elastography (SE). Imaging of strain and elastic modulus distributions in soft tissues based on external tissue compression, with subsequent computation of the strain profile along the transducer axis, was first described by Ophir *et al.*<sup>[1,2]</sup>. Strain imaging can be applied to the liver by inducing probe pressure<sup>[3]</sup>. The temporal derivative of strain, *i.e.*, the strain rate, is a measure of the rate of deformation<sup>[4]</sup>. Strain Rate Imaging is a Doppler-based method that can be used to mea-

sure strain of moving tissue<sup>[5,6]</sup>. The second form is shear wave elastography (SWE). Shear waves are generated in the tissues when a directional force is applied to the tissue which causes shear deformation. Shear waves are rapidly attenuated by tissue, they travel much more slowly (between 1 and 10 m/s) and they are not supported by liquids of low viscosity<sup>[7]</sup>.

The use of different ultrasound methods to estimate liver fibrosis have been published, such as transient elastography (TE) (FibroScan<sup>TM</sup>)<sup>[8-10]</sup>, strain elastography (*e.g.*, Hitachi Aloka Medical)<sup>[11-14]</sup> and SWE using acoustic radiation force impulse (ARFI) (Siemens *et al.*)<sup>[14-16]</sup>. Other techniques including 2D-SWE (Supersonic, Siemens) and 3D-SWE (Supersonic) have since been introduced<sup>[17-19]</sup>.

Recently the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) Guidelines and Recommendations have been published assessing the clinical use of ultrasound elastography<sup>[7,20]</sup>. The document is intended to form a reference and to guide clinical users in a practical way. The guidelines also give practical advice on its use and interpretation. Liver disease forms the largest section, reflecting the published experience to date, including evidence from meta-analyses with shear wave and strain elastography. This article comments on the EFSUMB elastography guidelines, discusses the multivariate factors that have an influence on LS, and the current techniques of ultrasound elastography as well as magnetic resonance elastography (MRE).

## LS AS A DYNAMIC AND MULTIFUNCTIONAL PROCESS

LS (elasticity) is a dynamic and multifunctional process. This means that factors influencing the stiffness and elasticity of a healthy liver are different to the factors in advanced fibrosis. However, many studies have examined the grade of liver fibrosis as the sole indicator of LS<sup>[21-25]</sup> (Table 1); a few others have evaluated more factors<sup>[26-29]</sup> (Table 1).

In patients with chronic liver disease, the assessment of the patient should include age, liver-related comorbidity, aetiology and duration of the liver disease, grading (inflammation), fatty infiltration, risk of malignant transformation, fibrosis, general comorbidity and many other factors. Such factors are important as they guide management and indicate prognosis. Therefore, the assessment of liver fibrosis is only one of many other important factors to determine before treatment. However, the focus on the assessment of liver fibrosis seems to be overstated and many studies lack the design of multivariate analysis.

Factors influencing liver elasticity in healthy subjects depend mainly on blood volume and perfusion parameters that are reported by surgeons during daily routine. Studies have reported a positive correlation of LS with central venous pressure<sup>[30]</sup>, therefore knowledge of co-existing cardiac and pulmonary disease is necessary for interpretation of results.

In addition, it is also reported that food intake could

significantly increase the LS in adults<sup>[31,32]</sup>, children<sup>[33]</sup> and the patients with chronic or resolved hepatitis C virus (HCV) infection<sup>[34]</sup>, therefore, elastography should be performed in fasting conditions. However, there is controversy on the influence of respiration on LS. Yun *et al.*<sup>[35]</sup> reported that LS was significantly elevated during expiration especially in patients without liver cirrhosis while Goertz *et al.*<sup>[32]</sup> did not find differences on the LS in deep inspiration, deep expiration and during Valsalva maneuver.

In liver cirrhosis, the degree and architecture of fibrosis is presumed to be the most important factor influencing LS (elasticity). The factors influencing liver elasticity in intermediate (significant) fibrosis are still not known in detail.

The factors influencing liver elasticity in patients with inflammatory disease (at least to some degree), independent of fibrosis, are acute hepatitis, any flare of transaminase values, acute-on chronic hepatitis<sup>[36,37]</sup>, cholestasis<sup>[38]</sup> and acute liver failure<sup>[39]</sup>. In a recent study of 104 patients with chronic hepatitis B (CHB) and 453 patients within chronic hepatitis C (CHC), histological necro-inflammatory activity was found to be an independent risk factor for the overestimation of LS in HCV and hepatitis B virus (HBV), while histological steatosis was a risk factor in HCV patients only<sup>[40]</sup>.

Other factors influencing liver elasticity in patients with fatty liver (hepatic steatosis) with or without inflammatory activity, with or without fibrosis, have also been described<sup>[41-47]</sup>.

The multivariate intercorrelation of factors influencing liver elasticity under different circumstances is not known. Since multiple factors have shown to influence LS measurements, interpretation of results has to be performed taking into account all these risk factors.

## DIAGNOSIS OF LIVER FIBROSIS

### Liver biopsy

Liver biopsy (LB) has been considered the “gold-standard” for grading and follow-up of necro-inflammatory activity and staging of fibrosis for more than fifty years<sup>[48,49]</sup>.

However, substantial limitations are obvious. Firstly, it is an invasive method with a significant complication rate<sup>[50]</sup>. A review of the literature regarding possible complications has recently been published<sup>[51]</sup>. Secondly, LB has shown some sampling variability<sup>[52]</sup>. The specimen obtained by LB represents a very small part of the liver (about 1/50000) but inflammatory and fibrotic activity is known to be patchy within the liver. The sampling variability can be reduced by mini-laparoscopic guided biopsy with the ability to evaluate the liver surface<sup>[53-57]</sup>, however, it has been shown that the sampling error using mini-laparoscopic guided biopsy is still about 30%<sup>[58]</sup>. LB has also shown some intra- and inter-observer variability<sup>[58,59]</sup>. Thirdly, there is a high inter-observer variability during microscopic evaluation<sup>[58]</sup>.

Therefore, one difficulty for the evaluation of non-

Table 1 Examples of studies

Title	Comment	Ref.
Univariate approach		
Elastographic assessment of liver fibrosis in children: A prospective single center experience	Pearson's correlation	[21]
Is it better to use two elastographic methods for liver fibrosis assessment?	Spearman rank correlation	[22]
Is ARFI elastography reliable for predicting fibrosis severity in chronic HCV hepatitis?	Spearman rank correlation	[23]
Factors that influence the correlation of acoustic radiation force impulse, elastography with liver fibrosis	Spearman rank correlation	[24]
Liver stiffness measurement using acoustic radiation force impulse elastography and effect of necroinflammation	Pearson product-moment correlation	[25]
Multivariate approach		
Liver stiffness measurements in patients with different stages of non-alcoholic fatty liver disease: Diagnostic performance and clinicopathological correlation	Spearman's correlation (no attention paid to Bonferroni or alpha correction) 6 factors (higher age, serum albumin, serum AST, serum cholesterol, diabetes mellitus, LSM), LSM is the only independent predictor of advanced fibrosis (odds ratio = 1.47, 95%CI: 1.23-1.77, $P < 0.001$ )	[26]
Assessment of liver fibrosis using transient elastography in patients with alcoholic liver disease	Spearman's correlation (with Bonferroni test). In multivariate analysis including fibrosis, HAH, and steatosis, fibrosis was the only histological parameter significantly correlated with LSM	[27]
FibroScan and ultrasonography in the prediction of hepatic fibrosis in patients with chronic viral hepatitis	Pearson correlation (no attention paid to Bonferroni or alpha correction) 12 factors. Multivariate analysis showed that LSM positively correlates with hepatic fibrosis, necro-inflammatory activity and ultrasound scores	[28]
Performance of unidimensional transient elastography in staging non-alcoholic steatohepatitis	Spearman's correlation (no attention paid to Bonferroni or alpha correction) 4 factors (fibrosis, ballooning, Lobular inflammation, steatosis). Multivariate analysis found fibrosis as the only factor influencing independently liver stiffness in NASH patients	[29]

LSM: Liver stiffness measurement; HAH: Hepatic abscess of hydatid origin; AST: Aspartate aminotransferase; NASH: Non-alcoholic steatohepatitis.

invasive markers of fibrosis is the use of LB as a reference method. Taking into account the limitations of LB, a perfect non-invasive method cannot be distinguished from an unacceptable fibrosis marker. Thus a new reference marker is needed. Studies have shown that non-invasive tests for liver fibrosis with FibroTest, enhanced liver fibrosis (ELF) and TE can predict 5-10 year survival of patients with CHC<sup>[60-64]</sup>. However, more studies using liver related mortality as the endpoint are still awaited to identify the best non-invasive methods<sup>[3]</sup>.

### Serum marker of liver fibrosis

One important non-invasive method for assessment of the severity of fibrosis includes serum markers<sup>[65-68]</sup>. So far, many serum biomarkers, both direct and indirect, have been evaluated for their ability to stage liver fibrosis<sup>[69-71]</sup>. Direct serum markers, reflecting either the deposition or the removal of extracellular matrix in the liver, include: (1) collagens such as type IV collagen, procollagen III N-peptide, collagenases; (2) inhibitors of collagens such as matrix metalloproteases and tissue inhibitory metalloprotease-1; and (3) glycoproteins such as serum hyaluronate, laminin, and YKL-40. So-called indirect markers include factors that can be measured from routine blood tests, such as platelet count, prothrombin index, and aspartate aminotransferase/alanine aminotransferase (AST/ALT), which indicate alterations in hepatic function. The usefulness of these markers has been assessed mostly in patients with CHC<sup>[70-72]</sup> and hyaluronate has been the most extensively studied direct marker<sup>[73,74]</sup>. These direct and indirect markers, when

used individually, are useful for the diagnosis or the exclusion of cirrhosis but have limited accuracy for the diagnosis of clinically significant fibrosis<sup>[75]</sup>. Therefore, more sophisticated algorithms or indices combining the results of groups of markers have been developed to improve the diagnostic accuracy. The FibroTest™ (proprietary formula; Biopredictive, Paris, France) was the first algorithm that combined these data<sup>[76]</sup>. Thereafter, several other indices, such as Fibrosure™ in the United States (LabCorp, Burlington, NC, United States), the Fibrometers™ (BioLiveScale, Angers, France), the FibroSpect II™ (Prometheus Laboratory Inc., San Diego, CA, United States), the ELF™ (Enhanced Liver Fibrosis Test, iQur Ltd, Southampton, United Kingdom) and the Hepascore™ (PathWest, University of Western Australia, Australia), have been developed. They are mainly for patients with CHC<sup>[77-80]</sup>, but can also be used in patients with hepatitis B<sup>[81,82]</sup> and human immunodeficiency virus (HIV)-HCV co-infection<sup>[83,84]</sup>. Among these indices, Fibrotest has been the one most extensively studied<sup>[69]</sup>.

In a prospective cohort of 537 HCV-infected patients, Fibrotest had a 5 year prognostic value (HCV-related complications and death) similar to that of LB<sup>[61]</sup>. In a meta-analysis<sup>[85]</sup> which included 6378 subjects with both FibroTest and biopsy (3501 HCV and 1457 HBV), the mean standardized area under the receiver operator curve (AUROC) for diagnosing significant fibrosis was 0.84 (95%CI: 0.83-0.86), without differences between HCV, 0.85 (95%CI: 0.82-0.87) and HBV, 0.80 (95%CI: 0.77-0.84). ELF has been evaluated in a recently published study<sup>[86]</sup> that included 196 patients. The ELF panel

had an AUROC of 0.90 for distinguishing severe fibrosis, 0.82 for moderate fibrosis, and 0.76 for no fibrosis, and it was improved to 0.98, 0.93 and 0.84, respectively, by the addition of simple markers. The clinical utility model showed that 82% and 88% of liver biopsies could potentially be avoided for the diagnosis of severe fibrosis using ELF and the combined panel, respectively<sup>[62,64]</sup>.

### Advantages and limitations

The practical advantages of analysing serum biomarkers to measure fibrosis include their high applicability and high inter-laboratory reproducibility<sup>[87,88]</sup>. However, the direct markers of liver fibrosis are not routinely available in most hospital settings, and none of the serum markers are liver specific—their results can be influenced by comorbidity. For example, FibroTest and Hepascore produce false-positive results in patients with Gilbert's syndrome or haemolysis as these patients have hyperbilirubinaemia<sup>[89]</sup>. Similarly, acute hepatitis can produce false-positive results in the marker measuring the level of aminotransferases, such as aspartate-to-platelet ratio index (APRI), Forns index, FIB-4, or Fibrometer tests.

### Magnetic resonance elastography

In recent years, magnetic resonance elastography (MRE) has been developed as a non-invasive functional magnetic resonance imaging (MRI) method for assessing and staging liver fibrosis, using a modified phase-contrast method to image the propagation characteristics of shear waves in the liver<sup>[90,91]</sup>. Elasticity is quantified by MRE and expressed in kilopascals (kPa) using a formula that determines the shear modulus, equivalent to one-third of the Young's modulus which is estimated with TE<sup>[72,92]</sup>. So far, there is only limited data on the accuracy of MRE. Several studies<sup>[92-96]</sup> have evaluated the usefulness of MRE for the assessment of LS among patients with chronic liver disease and have shown that increased shear stiffness measured on MRE is associated with increased severity of the fibrotic process. In addition, MRE has relatively high sensitivity and specificity for predicting the stage of hepatic fibrosis. It has shown at least equivalent diagnostic performance in fibrosis staging compared with TE with fewer limitations regarding its application in patients with a large amount of ascites or who are obese<sup>[92-95]</sup>. Yin *et al.*<sup>[95]</sup> reported sensitivity of 86% and 78%, and specificity of 85% and 96%, with cut-off values of 4.89 and 6.47 kPa, respectively. Huwart *et al.*<sup>[93]</sup> showed similarly high sensitivity of 98% and 95%, and specificity of 100% and 100%, for discrimination, but lower cut-off values of 2.5 and 3.1 kPa were used. The reason for the difference in cut-off values obtained in the two studies may potentially be explained by the differently manufactured scanners used for MRE acquisition, case mixes, imaging protocols, and post-processing procedures. A meta-analysis has been recently published<sup>[97]</sup>.

### Advantages and limitations

Compared with TE, dynamic MRE has the potential

to assess larger volumes (almost the entire liver) and to provide full three-dimensional information about the viscoelastic properties of tissues<sup>[98]</sup>, moreover, due to the theoretical advantages, MRE is capable of application to patients with obesity or ascites. However, MRE cannot be performed on the liver of patients with iron overload because of signal-to-noise limitations and it is too costly and time-consuming to use in routine practice<sup>[72]</sup>.

## INTRODUCTION TO ULTRASOUND ELASTOGRAPHY

### TE

TE (FibroScan<sup>®</sup>) was the first tool introduced for routine clinical use (Echosens, Paris, France) (Figure 1). TE does not display a conventional ultrasound image. TE has been mainly evaluated in patients with chronic viral hepatitis C and also in a few patients with HIV/viral hepatitis C co-infection and some other liver diseases (see below)<sup>[99]</sup>.

### Technique

**Basic principles:** TE is an ultrasound-based non-invasive method. It is characterized by the material's strain response to external stress according to the principle of Hooke's law<sup>[9]</sup>. Briefly, an ultrasound transducer probe is mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency (50 Hz) are transmitted by the transducer from a right intercostal space, inducing an elastic shear wave that propagates through the liver. Pulse-echo ultrasound acquisition is used to follow the propagation of the shear wave and to measure its speed. This speed is proportional to the tissue stiffness, with faster wave progression occurring through stiffer material. The elastic modulus  $E$  is expressed as  $E = 3qV^2$ , where  $V$  is the shear wave speed and  $q$  is the material density (assumed constant for tissues): the stiffer the tissue, the faster the shear wave propagates<sup>[100]</sup>. TE measures LS in a cylindrical volume approximately 10 mm wide and 40 mm long, between 25 and 65 mm below the skin surface with the standard M-probe, and between 35 and 75 mm for the recently developed XL probe, recommended for obese patients<sup>[101,102]</sup>. This volume is at least 100 times bigger than a biopsy sample and it has been suggested, therefore, that the results compared to LB are more representative of the hepatic parenchyma. However, TE does not work for the left liver lobe or from a subcostal approach and the measurement is only feasible *via* a few intercostal spaces. Therefore, the technique is limited. Inter- and intra-observer variability depend on the intercostal space used, the presence of ascites, musculoskeletal habitus, depth of subcutaneous tissue, position of the patient, and many other factors<sup>[47,103]</sup>.

**How to perform?** The measurements with FibroScan<sup>®</sup> are taken from the right liver lobe *via* an intercostal space, while the patient lies flat on his/her back, with the right arm tucked behind the head to facilitate access to the liver parenchyma. The tip of the probe is covered with

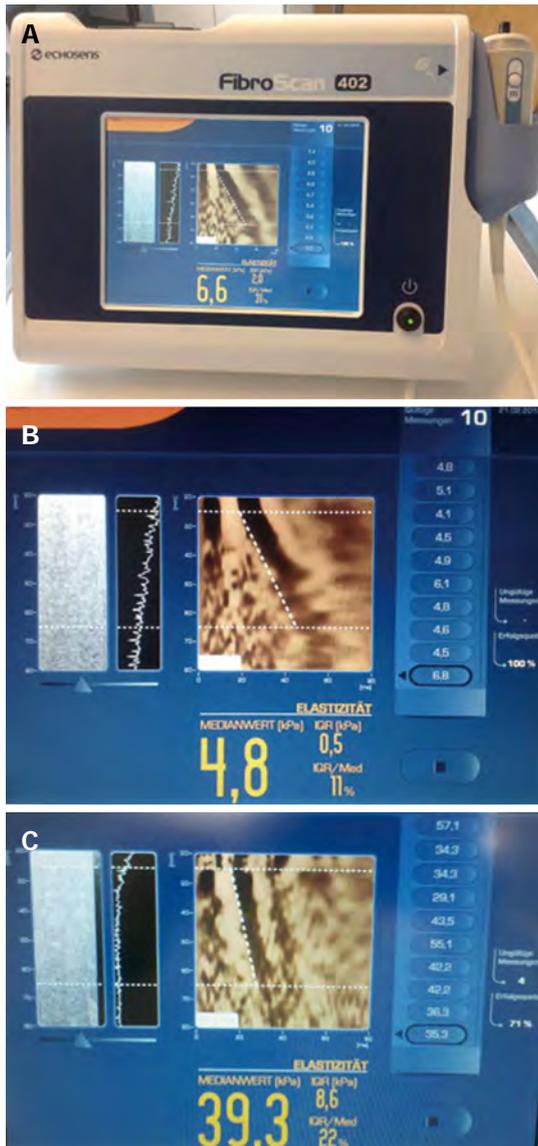


Figure 1 Transient elastography (FibroScan<sup>®</sup>, A) for the evaluation of normal liver (B) and liver cirrhosis (C).

coupling gel and placed on the skin between the ribs at the level of the right lobe where LB would be performed. Once the measurement area has been located, the operator presses the probe button (shot) to start an acquisition. When a shot is unsuccessful, the machine does not give a reading. Measurement of LS is measured in kilopascals (kPa) (range is between 2.5 and 75 kPa)<sup>[100]</sup>.

**Advantages:** TE with FibroScan<sup>®</sup> is a rapid procedure (less than 5 min), painless, and easy to perform even in the outpatient clinic or at the bedside. The results are immediately available<sup>[103]</sup>. The examination can be performed by a nurse after a short learning curve (about 100 examinations)<sup>[104]</sup>. In addition, TE analysis has excellent inter- and intra-observer agreement, which makes it suitable for widespread application in clinical practice<sup>[103,105,106]</sup>.

**Limitations:** TE provides only a regional elasticity measurement (determined by the width of the ultrasound beam and depth of the shear wave penetration), but no anatomical images or elastograms. Other drawbacks include limited depth (several cm), the inability of the shear wave to propagate beyond fluid collections (ascites) and difficulty in obtaining sufficient signal in obese patients. Recently, a new probe (XL probe; Echosens, Paris, France) has been proposed for overweight and obese patients<sup>[107]</sup>, and a so-called S-probe has been developed for patients with narrow intercostal spaces, especially children<sup>[108]</sup>. However, it remains impossible to obtain TE results from patients with ascites<sup>[105]</sup>.

The validity of the TE result also depends on two important parameters: (1) the success rate (the ratio of the number of successful measurements to the total number of acquisitions) should be at least 60%; and (2) the interquartile range (IQR), which reflects the variability of the validated measurements, should not exceed 30% of the median value<sup>[109]</sup> (Figure 1). Both the feasibility and reproducibility of the TE measurement may be affected by high body mass index (BMI). In a study with 13369 TE measurements, a failure rate of 3.1% was reported. Unreliable results were reported in 15.8% of measurements and were associated with a BMI > 30 kg/m<sup>2</sup>, age > 52 years, female sex, operator experience and type 2 diabetes<sup>[47]</sup>.

The clinical interpretation of TE results should always be made by an expert clinician and with reference to the patient's history, disease aetiology and essential laboratory parameters Castera *et al*<sup>[110]</sup>.

**Intra- and inter-observer variability:** Several studies<sup>[103,105,106]</sup> have shown that the intra- and inter-observer reproducibility of TE measurements are good, at least in non-obese subjects. In the study by Sandrin *et al*<sup>[105]</sup> intra- and inter-observer variation in TE was investigated in 15 patients and was around 3%, but with a wide variation (2%-18%). The sample size of this study was small, and therefore inadequate to draw firm conclusions on host- and disease-related co-variables that may interfere with TE performance. Another study by Fraquelli *et al*<sup>[103]</sup> with a larger sample obtained similar results; 800 TE examinations were performed by two operators in 200 patients with various chronic liver diseases. Both inter- and intra-observer agreement was high and TE reproducibility was excellent, with an intraclass correlation coefficient (ICC) of 0.98. However, inter-observer agreement was significantly reduced in patients with mild hepatic fibrosis, and hepatic steatosis.

The probe location during the TE measurement may affect its reproducibility. In a recent study<sup>[111]</sup> TE was performed on 625 consecutive patients with chronic liver disease at three different sampling sites. Sampling variability according to probe location was seen in approximately 30% of patients and it was suggested that TE should be performed from various sites to minimize the sampling error.

### Review of the literature

**Chronic viral hepatitis:** For patients with CHC, LS values  $> 6.8$ - $7.6$  kPa are indicative of significant fibrosis ( $F \geq 2$ ) using the gold standard of LB, and the cut-off values for predicting complete cirrhosis ( $F = 4$ ) range between 11.0 and 13.6 kPa<sup>[20,112,113]</sup>. TE is able to distinguish mild fibrosis from advanced liver fibrosis and cirrhosis, which is important for decision making<sup>[114]</sup>. In contrast, TE does not allow differentiation between the contiguous stages of liver fibrosis. In a meta-analysis including 40 studies<sup>[114]</sup>, the pooled sensitivity and specificity of TE was 79% and 78% for the diagnosis of significant fibrosis; 82% and 86% for diagnosing severe fibrosis; and 83% and 89% for the diagnosis of liver cirrhosis.

It might be of interest to remember that conventional ultrasound techniques can also distinguish between liver cirrhosis and early liver disease in approximately 70% of patients with high specificity but low sensitivity<sup>[115-124]</sup>. However, TE had an acceptable diagnostic accuracy for detecting early compensated cirrhosis in patients with CHB who did not fulfil the clinical and ultrasound criteria for cirrhosis<sup>[125]</sup>. Conventional ultrasound techniques are helpful in the detection of complications of liver cirrhosis including portal hypertension<sup>[126,127]</sup> and can also give important information about fatty infiltration<sup>[128-132]</sup> and inflammation<sup>[133-136]</sup>. In a study with 90 patients with suspected liver cirrhosis, liver surface nodularity on conventional ultrasound and TE showed comparable results for diagnosis and exclusion of liver cirrhosis, with the best results when both methods were combined. Liver surface nodularity was better for the diagnosis of liver cirrhosis, while TE was better at ruling out cirrhosis<sup>[137]</sup>.

The performances of TE, when compared, have been shown to be similar between patients with HBV and HCV<sup>[138]</sup>. Several studies have investigated the performance of TE in an Asian population with CHB<sup>[125,139-144]</sup> and concluded that TE is a promising and accurate tool for the early detection of cirrhosis. It is demonstrated that the optimal cut-off values for diagnosing HBV-related cirrhosis were between 9.0 and 10.1 kPa in the Asian population<sup>[125,140,142,145]</sup>, which is lower than that in patients with CHC<sup>[146,147]</sup>. Since there is an increasing number of evidence on the usefulness of TE in patients with CHB, especially in the Asian population, TE should also be recommended in patients with CHB, though the evidence is more limited compared to CHC. Future and updated guidelines have to include this recommendation.

It would be interesting to know in what percentage of patients TE can give important additional information which is of relevance to the treatment, over and above sophisticated ultrasound technology in the hand of an expert hepatologist<sup>[43,148]</sup>.

### EFSUMB recommendations

TE can be used to assess the severity of liver fibrosis in patients with chronic viral hepatitis, provided that confounding factors are taken into account, and especially to distinguish patients with nil/mild fibrosis from those with

significant fibrosis, and to identify those with cirrhosis. TE is useful for assessment of liver fibrosis in patients with non-alcoholic fatty liver disease (NAFLD), alcoholic liver diseases, and in patients co-infected with HIV and HCV. Other types of chronic liver disease might also have been investigated, but the evidence is more limited. TE is useful for assessment of liver fibrosis in patients with post-transplant recurrence of CHC. TE has some value for predicting the occurrence of complications of liver cirrhosis, portal hypertension, hepatocellular carcinoma (HCC) and liver-associated mortality. It cannot replace upper gastrointestinal endoscopy for identifying patient with varices<sup>[20]</sup>.

## POINT SHEAR WAVE ELASTOGRAPHY WITH ACOUSTIC RADIATION FORCE IMPULSE IMAGING

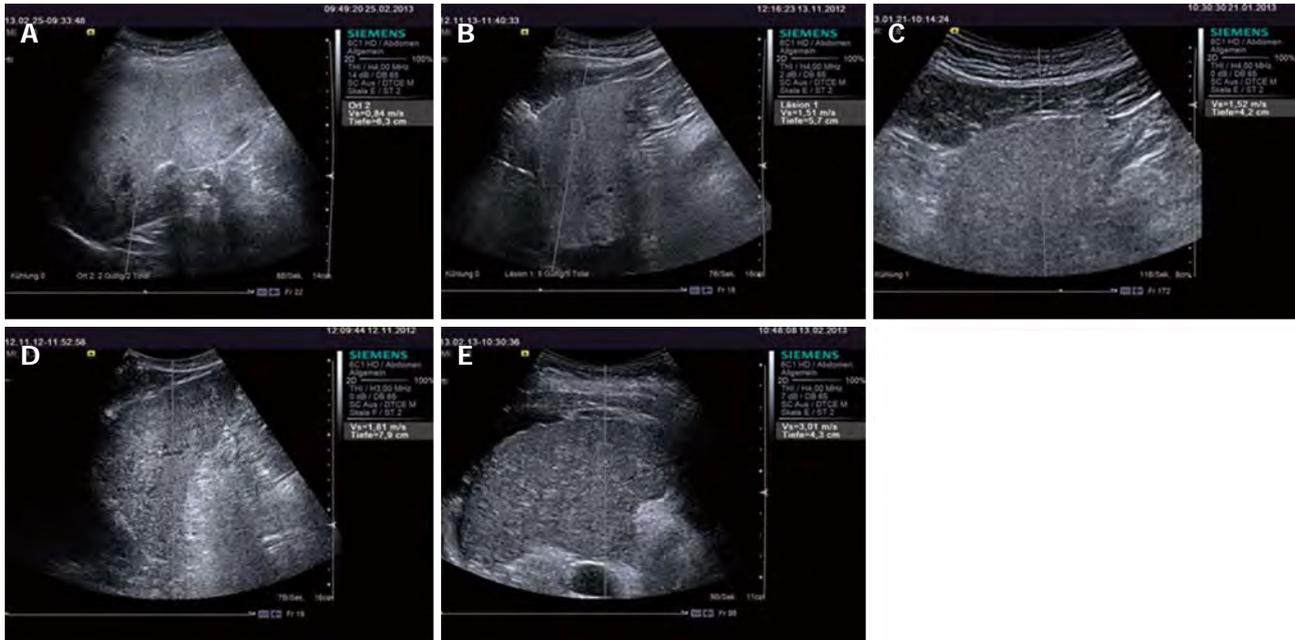
Point shear wave elastography (pSWE) has been introduced by different companies, each currently at different stages of development<sup>[7,20]</sup>. Acoustic radiation force impulse (ARFI) was the second method to be introduced as a tool for liver fibrosis assessment in a clinical setting. ARFI has a significant advantage over TE in that it simultaneously displays a conventional ultrasound image. The accuracy of both methods has been shown to be similar in the differentiation of normal liver parenchyma from liver cirrhosis<sup>[15,149,150]</sup>. ARFI has been mainly evaluated in patients with chronic viral hepatitis C and in a few other liver diseases.

### Technique

**Basic principles:** ARFI quantification has been developed by two companies (Siemens and Philips) according to the guidelines<sup>[7,20]</sup>, almost all reported studies were done with a conventional high-end ultrasound machine (Siemens S2000). It uses a region of interest (ROI) cursor to interrogate the elastic properties of a specific anatomic region, while real-time B-mode imaging of the abdomen being performed. Short-duration acoustic pulses with a fixed transmit frequency of 2.67 MHz, are generated in the vicinity of the ROI and the subsequent mechanical excitation of the tissues results in tissue displacement and the formation of shear waves that propagate away from the region of excitation. Ultrasound tracking beams laterally adjacent to the single push-beam are used to estimate the shear wave speed in the tissue by the measurement of the time to peak displacement at each lateral location<sup>[151]</sup>. The shear wave speed is estimated in the central window 5 mm long by 4 mm wide within a graphically displayed ROI of size 10 mm long by 6 mm wide. The results are expressed in meters per second (m/s) (range: 0.5-4.4 m/s with  $\pm 20\%$  accuracy over the range), the shear wave propagation speed being proportional to the square root of the tissue elasticity<sup>[152,153]</sup>. The ARFI imaging examination takes approximately 5 min. Unlike FibroScan<sup>®</sup>, ARFI can be utilized in patients with ascites. No limitations

**Table 2 Mean shear wave velocities (VirtualTouch values) of the left and right liver lobes (mean  $\pm$  SD)**

Ref.	n	Subjects	Left lobe (m/s)	Right lobe (m/s)
Karlas <i>et al.</i> <sup>[158]</sup>	50	Healthy individuals	1.28 $\pm$ 0.19	1.15 $\pm$ 0.17
Karlas <i>et al.</i> <sup>[158]</sup>	23	Patients with F1, F2 fibrosis	2.1 $\pm$ 0.73	1.75 $\pm$ 0.89
Toshima <i>et al.</i> <sup>[159]</sup>	103	24 healthy volunteers, 79 patients with chronic liver disease	1.90 $\pm$ 0.68	1.61 $\pm$ 0.51
Piscaglia <i>et al.</i> <sup>[157]</sup>	14	Healthy individuals	1.29 (1.00-1.60)	1.15 (0.80-1.74)
Piscaglia <i>et al.</i> <sup>[157]</sup>	114	Patients with chronic liver disease	1.79 (0.80-4.00)	1.67 (0.45-3.76)

**Figure 2** Point-shear wave elastography with acoustic radiation force impulse for the evaluation of histologically proved liver fibrosis and cirrhosis. A: F0; B: F1; C: F2; D: F3; E: F4 = cirrhosis.

concerning measurement are known<sup>[154]</sup>.

**Tips and tricks:** When scanning the right lobe (especially segment VIII), an optimal window should be used. To reduce the variance of the measurement, it is recommended to apply minimal scan pressure and for the patient to minimize breathing, the influence of cardiac motion should also be avoided. In general, the best and most consistent results will occur when the “normal” state of the liver is measured. When scanning intercostally, no pressure should be applied to the liver and the patient should be asked to just stop breathing for a moment (instead of deep inspiration and breath hold).

In difficult patients, several measurement attempts are needed to “average” out the readings, and data that varies significantly should be excluded. It is recommended to put the patient in a left lateral decubitus position with right arm behind the head in order to get better access to the liver without excessive pushing or the need for breath holds<sup>[155]</sup>. However, it may still not be possible to get reliable readings in 5.3 % of patients<sup>[156]</sup>.

**Intra- and inter-observer variability:** Reproducibility of ARFI is also an important pre-requisite for its widespread application in clinical practice. Good inter-observer vari-

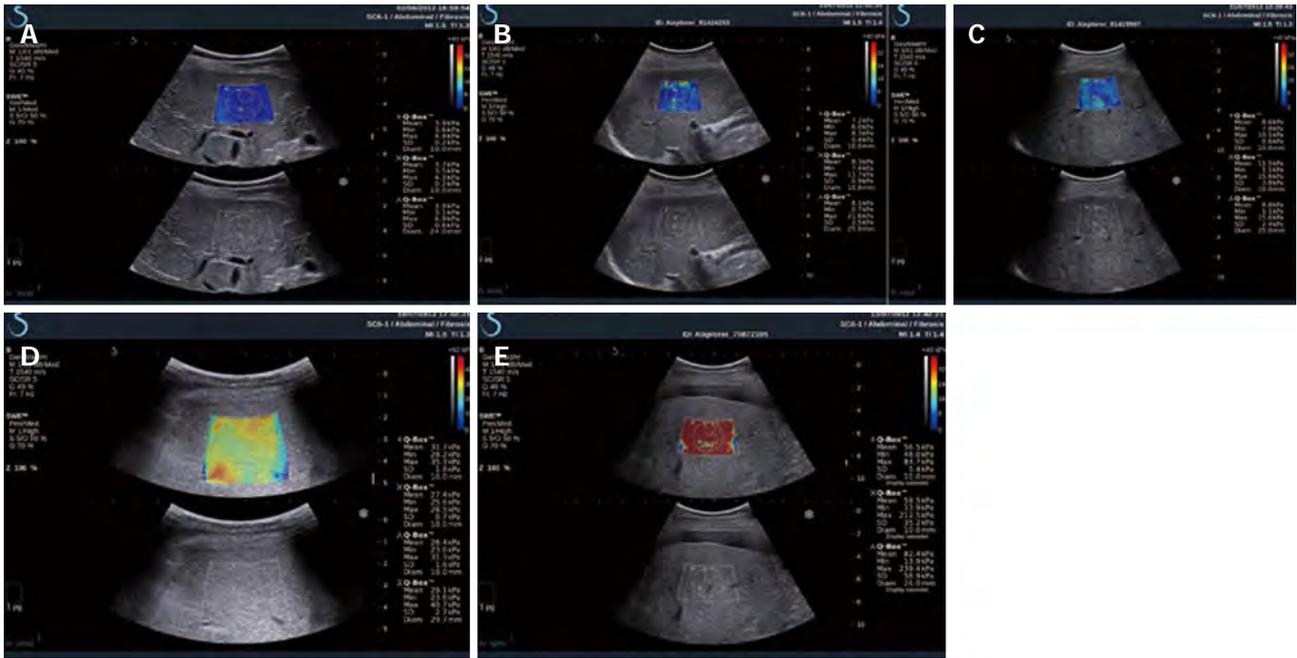
ability has been reported<sup>[157]</sup>. Since ARFI allows different measurement sites, comparison of measurements in the right and left liver lobes have been made, and have shown a trend toward higher values in the left lobe<sup>[157-159]</sup>. However, results in the right lobe revealed higher diagnostic accuracy compared to the left (AUROC: for diagnosis of F1, F2, F3, F4, right lobe: 0.92; 0.83; 0.86; 0.80; left lobe: 0.77; 0.71; 0.78; 0.84; sensitivity, specificity, positive predictive value and negative predictive value of right lobe: 0.88; 0.81; 0.74; 0.92; left lobe: 0.80; 0.75; 0.87; 0.68)<sup>[159]</sup> (Table 2).

### Clinical applications

**Chronic viral hepatitis:** In patients with significant fibrosis ( $F \geq 2$ ) the ARFI cut-off values published have been between 1.21-1.34 m/s (AUROCs 0.85-0.89)<sup>[23,149,150]</sup> and in patients with cirrhosis, 1.55-2 m/s (AUROC's 0.89-0.93)<sup>[15,23,149,160]</sup> (Figure 2). Similar to TE, SWE has not proved accurate enough to distinguish between contiguous stages of fibrosis<sup>[20]</sup>.

**Other liver diseases:** ARFI has also been evaluated in patients with NAFLD and NASH<sup>[161,162]</sup> and in patients after liver transplantation<sup>[163]</sup>.

**Meta-analysis:** Friedrich-Rust *et al.*<sup>[154]</sup> published a meta-



**Figure 3** 2D-shear wave elastography with supersonic shear imaging for the evaluation of histologically proved liver fibrosis and cirrhosis. A: F0; B: F1; C: F2; D: F3; E: F4 = cirrhosis.

analysis which included 9 studies with a combined total of 518 patients with chronic liver disease and evaluated the diagnostic performance of ARFI imaging for the staging of liver fibrosis. The diagnostic accuracy of ARFI quantified by the AUROC was 87% for predicting significant fibrosis ( $F \geq 2$ ), 91% for the diagnosis of severe fibrosis ( $F \geq 3$ ) and 93% for the diagnosis of liver cirrhosis. The meta-analysis revealed good diagnostic accuracy for ARFI in the diagnosis of significant liver fibrosis and excellent diagnostic accuracy for the diagnosis of liver cirrhosis.

It was also shown that a comparison of ARFI with TE in the four studies that included 312 patients, resulted in comparable diagnostic accuracies for both methods in the diagnosis of severe fibrosis, and slightly, but significantly, higher diagnostic accuracies of TE for the diagnosis of significant fibrosis and liver cirrhosis. However, a recent study showed superior results for ARFI elastography<sup>[150]</sup>. Future multicentre studies are necessary to compare the different methods before any conclusions can be drawn.

#### Advantages and limitations

In contrast to TE, ARFI has been shown to be less influenced by obesity and ascites<sup>[151]</sup>. One study showed that valid LS measurement (LSM) were obtained in all 23 patients with morbid obesity (mean BMI was higher than  $44 \text{ kg/m}^2$ )<sup>[164]</sup>. In addition, it can be easily added to a commercial ultrasound machine.

However, in contrast to TE values, ARFI values have a narrow measurement range (0.5-4.4 m/s), which limits the definition of cut-off values required for decisions on patient management. In addition, inflammatory activity

and elevated aminotransferase levels may lead to overestimation of ARFI-LS values<sup>[15,25]</sup> as has been shown for TE. Moreover, since this is a new technique, the quality criteria are not yet well-defined.

#### EFSUMB recommendations

pSWE with ARFI can be used to assess the severity of liver fibrosis in patients with chronic viral hepatitis, especially with hepatitis C. pSWE with ARFI is promising for liver fibrosis assessment in patients with NAFLD, and post-transplant patients<sup>[7,20]</sup>.

## 2D SWE

SWE [Aixplorer<sup>®</sup>, SuperSonic Imagine (SSI), France] has been introduced as a 2D and also 3D-technique. So far, only 2D-SWE has been evaluated in studies on the liver. The studies of 3D-SWE have mainly focused on the breast<sup>[165,166]</sup>.

#### Technique

This technique is based on the combination of a radiation force induced in the tissues by focused ultrasonic beams and very high frame rate (up to 5000 f/s) ultrasound imaging capable of catching, in real time, the transient propagation of the resulting shear waves<sup>[167,168]</sup>. The local shear wave speed is recovered using a dedicated time-of-flight estimation technique and enables the 2-D quantitative mapping of elasticity. This imaging modality can be performed using a conventional ultrasound probe, during a standard intercostal ultrasound examination. Three supersonic shear wave imaging sequences are applied successively to the left, middle and right parts of

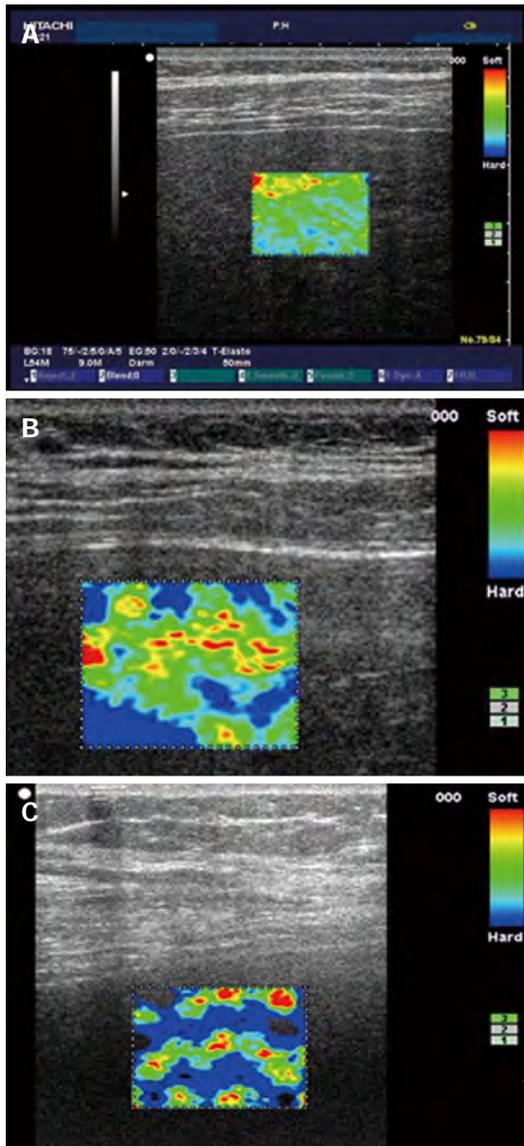


Figure 4 Strain elastography for the evaluation of histologically proved liver fibrosis and cirrhosis. A: F0; B: F2; C: F4 = cirrhosis.

the 2-D ultrasound image. The resulting elasticity images in the three regions are concatenated to provide the final image covering the entire region-of-interest. The ability of the SWE technique to provide a quantitative and local estimation of liver shear modulus with a millimetric resolution has been proven in a pilot study in 15 healthy volunteers<sup>[18]</sup>. Liver moduli extracted from *in vivo* data from healthy volunteers, were consistent with those reported in the literature (Young's modulus ranging from 4 to 7.5 kPa). Moreover, LSM using the SWE mode was fast (less than one second), repeatable (5.7% standard deviation) and reproducible (6.7% standard deviation)<sup>[3]</sup>.

**Intra- and inter-observer variability:** To date, there has only been one study<sup>[169]</sup> aimed at assessing the intra- and inter-observer precision of 2D-SWE measurements in the evaluation of liver elasticity. It was reported that the

reproducibility was good with high intra- and inter-observer agreement. In this study, 2D-SWE was performed on 60 volunteers (42 cases with 10 consecutive measurements, 18 cases with 2 measurements) on 2 different days by 2 operators (one expert and one novice). The intra-observer agreement between measurements performed in the same subject on the same day (day 1 or day 2) showed intraclass correlation coefficient (ICC) values of 0.95 and 0.93 for the expert operator and novice, respectively, and the ICC values for intra-observer agreement between measurements performed in the same subject on different days were 0.84 and 0.65, respectively. The inter-observer agreement was 0.88. Therefore, real-time 2D-SWE has been shown to be a reproducible method to measure liver elasticity, but the novice operator showed lower measurement reproducibility over time than the expert operator.

### Clinical application

**CHC:** 2D-SWE might be used in assessing liver fibrosis for patients with CHC, as has been proved in two large studies. Ferraioli *et al.*<sup>[170]</sup> assessed the accuracy of 2D-SWE in comparison with transient elastography in 121 patients with CHC using LB as the reference standard, and found that LS values increased in parallel with the degree of liver fibrosis both with 2D-SWE and TE. The AUROC was 0.92 for 2D-SWE and 0.84 for TE ( $P = 0.002$ ); 0.98 for 2D-SWE and 0.96 for TE ( $P = 0.14$ ); 0.98 for 2D-SWE and 0.96 for TE ( $P = 0.48$ ), when comparing F0-F1 *vs* F2-F4, F0-F2 *vs* F3-F4, and F0-F3 *vs* F4, respectively (Figure 3). Therefore, the real-time 2D-SWE was more accurate than TE in assessing significant fibrosis ( $\geq F2$ ). In the other study which included 113 hepatitis C virus patients, a good agreement was shown between 2D-SWE and TE, the AUROC for elasticity values assessed by 2D-SWE were 0.948, 0.962 and 0.968 for patients with predicted fibrosis levels  $F \geq 2$ ,  $F \geq 3$  and  $F = 4$ , respectively. However, LB was only available in 39 patients<sup>[17]</sup>.

### EFSUMB recommendations

2D-SWE can be used to assess the severity of liver fibrosis in patients with chronic viral hepatitis, especially with hepatitis C<sup>[7,20]</sup>.

## STRAIN ELASTOGRAPHY

Strain elastography (SE), also termed as quasi-static strain imaging, has been developed by several manufacturers, however, only Hitachi ultrasound system has been evaluated for use in liver.

### Technique

SE is based upon the fact that soft tissue can be more easily compressed than hard tissue. When subtle compression is applied with probe, SE shows the relative degree of tissue strain, but not demonstrates the physical elasticity directly. SE calculates the strain response of the tissue

**Table 3** Advantages and disadvantages of non-invasive methods to evaluate liver fibrosis

Parameters	Transient elastography	ARFI	2D-SWE	MR Elastography	Serum biomarkers
Advantages	High and rapid performance Reproducibility Easy to learn	High and rapid performance Reproducibility Easy to learn	High and rapid performance Reproducibility Easy to learn, large ROI	High performance (applicability) Reproducibility Examination of the whole liver	Availability Reproducibility Low cost
Disadvantages	Technical requirements (equipment) without additional use Intermediate cost Limited recognition of intermediate stages of fibrosis Blind selection of region of interest Restricted value in obese patients and ascites False positive values in patients with acute hepatitis, cholestasis, and heart failure	Combined with conventional ultrasound Obesity and ascites are not limiting Technical requirements (ultrasound equipment) Intermediate cost Limited recognition of intermediate stages of fibrosis Narrow range of values, small ROI Quality criteria not well defined	Combined with conventional ultrasound Ascites are not limiting Technical requirements (ultrasound equipment) Intermediate cost Limited recognition of intermediate stages of fibrosis Quality criteria not well defined	Combined with conventional MRI obesity and ascites are not limiting Technical requirements (MRI equipment) Extremely high cost, time consuming Limited recognition of intermediate stages of fibrosis Not applicable in case of iron deposition	Non-specific (hyperbilirubinemia, hemolysis, inflammation, others) Relatively high cost, limited availability (patent) Limited recognition of intermediate stages of fibrosis Results not immediately available

ARFI: Acoustic radiation force impulse; SWE: Shear wave elastography; ROI: Region of interest; MRI: Magnetic resonance imaging.

to stress (relative tissue elasticity) and displays it as a colour overlay [ranges from red (soft) to blue (hard)] on the B-mode image<sup>[171]</sup>. The echo signals could be captured in real-time by incorporating a high speed algorithm, in addition, both the B-mode image and corresponding tissue elasticity image could be simultaneous displayed<sup>[172]</sup>. Semi-quantitative elastography techniques are based on quantification of the strain distribution within a defined ROI.

Because the pressure generated by the operator's compression may influence both the image of elasticity and the resulting elasticity score, Hitachi medical system has recently developed an elastography method that did not require extra external stress. The required liver distortion for future analysis would be achieved from the rhythmic pulsations of the abdominal aorta or the heart.

### Clinical application

In 2007, Frederich-Rust *et al*<sup>[111]</sup> reported the clinical application of SE in the liver. They developed an elasticity score by assessing the colour-coded strain image using the computer program Matlab. The diagnostic accuracy for F2, F3 and F4 were 0.75, 0.73 and 0.69, respectively. In 2009, the same group<sup>[173]</sup> compared SE with TE (Fibroscan) and serum fibrosis marker (Fibrotest), and concluded that SE in its evaluated format could not replace TE for non-invasive assessment of liver fibrosis at the time of the study. After the software for elastography was developed by Hitachi medical systems, good results were published by several studies. Morikawa *et al*<sup>[174]</sup> transferred the pixel data in the ROI into a histogram and a binary

image for semi quantification with a devised system, and found that the mean value on the histogram and the percentage of hard tissue may directly represent liver elasticity. The diagnostic accuracy of SE for liver fibrosis was also compared with TE, the author felt SE compared favourably with TE and suggested SE could potentially be used as a routine imaging tool to evaluate liver fibrosis. A Chinese group<sup>[175]</sup> utilized a new Hitachi ultrasound system (HI VISION Preius) and concluded that there was a strong positive correlation ( $r = 0.81$ ) between the elasticity index and fibrosis stage. Diagnostic accuracies of SE for the diagnosis of F1, F2, F3, F4 were 0.93, 0.92, 0.84 and 0.66, respectively. Koizumi *et al*<sup>[176]</sup> performed a semi-quantitative analysis using the elastic ratio method (ratio of strain distribution in two selected ROI) on 70 patients with CHC and with the hepatic vein as the internal control and found that the AUROC curves for elastic ratio were superior to serum fibrosis markers and scores of fibrotic change based on blood results (Figure 4).

More studies including meta-analysis about the use of SE for the evaluation of liver fibrosis are required to establish a protocol for accurate imaging and to standardize analysis.

### Advantages

The main advantage of this technique is the relatively large region of interest that can be interrogated in the right liver lobe, plus the quantification method that can measure the change from the diffuse soft uniform architecture of the liver to a patchy hard pattern as hepatic

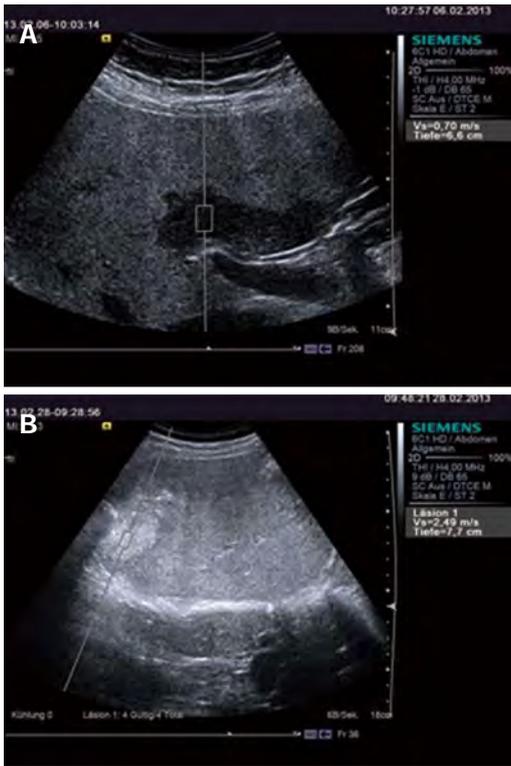


Figure 5 Point-shear wave elastography with acoustic radiation force impulse for evaluation of focal liver fatty lesion (A) and liver metastasis (B).

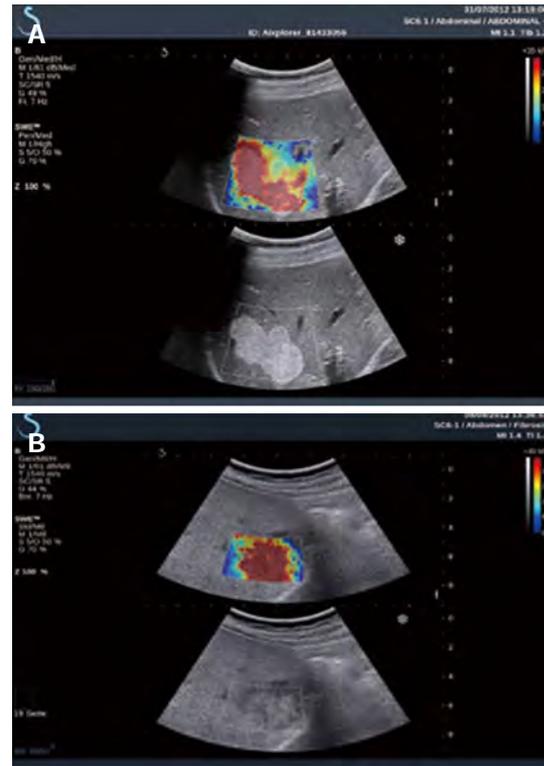


Figure 6 2D shear wave elastography with supersonic shear imaging for the evaluation of liver hemangioma (A) and metastasis (B).

**Table 4 Performance of acoustic radiation force impulse in the identification of malignant focal liver lesions**

No. of FLL	Rate of malignancy	Reference standard	Lesion types	ARFI cut-off (m/s)	QUADAS score	Ref.
105	64.8%	Biopsy, imaging	Haemangioma, FNH, focal fatty sparing, focal fat deposits adenomas, HCC, metastasis	2.7	11	[182]
60	71.7%	Biopsy, CT/MRI	haemangioma, HCC, CCC, metastasis	2	10	[183]
128	53.1%	Biopsy, surgery, imaging	Haemangioma, FNH, focal fatty change, abscess, adenoma, solitary necrotic nodule, HCC, metastasis, CCC	2.2	10	[184]
42	64.3%	Biopsy	Haemangioma, lymphoma, FNH, sarcoid, abscess, focal fatty sparing, HCC, metastasis	2.5	12	[185]
45	22.2%	Biopsy, CT/MRI	Haemangioma, metastasis	2.5	8	[186]

QUADAS: Quality assessment of diagnostic accuracy studies; HCC: Hepatocellular carcinoma; FNH: Focal nodular hyperplasia; CCC: Cholangiocarcinomas; FLL: Focal liver lesions; CT: Computed tomography; MRI: Magnetic resonance imaging.

fibrosis progresses.

**Intra- and inter-observer reproducibility**

The intra-observer variability and intra-observer agreement of SE for the assessment of liver fibrosis have been criticized in several studies<sup>[173,177,178]</sup>. In a more recent study, a Japanese group<sup>[176]</sup> used a semi-quantitative method (elastic ratio) and found that the measurements obtained from four separate locations had no observed variation between the two operators ( $K = 0.835$ ,  $ICC = 0.966$ ).

**EFSUMB recommendations**

The evidence with this approach is still too limited to allow recommendation for its clinical use, at least in European patients<sup>[7,20]</sup>.

**ADVANTAGES AND DISADVANTAGES OF CURRENT NON-INVASIVE METHODS IN EVALUATING LIVER FIBROSIS**

Advantages and disadvantages of currently available non-invasive methods in patients with chronic viral hepatitis C are summarized in Table 3.

**ELASTOGRAPHY FOR DETECTION AND CHARACTERIZATION OF FOCAL LIVER LESIONS**

Elastography methods have been also applied for detection and characterisation of focal liver lesions (FLL).

Although the method so far cannot be applied to all segments and the limited depth of penetration is so far disappointing, several studies have evaluated the performance of ARFI to differentiate FLL, and the results are encouraging. ARFI has shown a high accuracy for the identification of malignant FLL. In a meta-analysis by Ying *et al.*<sup>[179]</sup> including 590 lesions in eight studies, the summary sensitivity and specificity for identification of malignant liver lesions were 0.86 and 0.89, respectively. The hierarchical summary receiver operating characteristic (HSROC) was 0.94. However, one paper showed that ARFI did not permit differentiation between benign and malignant FLL because high ARFI values occur in benign as well as in malignant lesions<sup>[180]</sup>. In another study by Gallotti A, the mean shear wave speed of HCC, haemangioma, adenoma, metastasis, FNH was 2.17, 2.30, 1.25, 2.87, 2.75 m/s, respectively. Adenoma showed similar stiffness to the surrounding liver, and was significantly softer than the other four types of lesion. FNH showed different stiffness to HCC and metastasis, however, haemangioma showed no difference to HCC, metastasis and FNH<sup>[181]</sup> (Figures 5 and 6). The performance of ARFI is summarized in Table 4.

#### EFSUMB recommendations

Although promising results have been reported, more research is needed, especially in comparison to CEUS, before recommendations on its use in clinical practice can be made. So far, elastography cannot be recommended for the differential diagnosis of benign from malignant liver lesions<sup>[7,20]</sup>.

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## Hepatectomy for bile duct injuries: When is it necessary?

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### Abstract

Iatrogenic bile duct injuries (IBDI) are still a challenge for surgeons. The most frequently, they are caused by laparoscopic cholecystectomy which is one of the commonest surgical procedure in the world. Endoscopic techniques are recommended as initial treatment of IBDI. When endoscopic treatment is not effective, surgery is considered. Different surgical biliary reconstructions are performed in most patients in IBDI. Roux-Y hepaticojejunostomy is the commonest biliary reconstruction for IBDI. In some patients with complex IBDI, hepatectomy is required. Recently, Li *et al* analyzed the factors that had led to hepatectomy for patients with IBDI after laparoscopic cholecystectomy (LC). Authors concluded that hepatectomy might be necessary to manage early or late complications after LC. The study showed that proximal IBDI (involving hepatic confluence) and IBDI associated with vascular injuries were the two independent risk factors of hepatectomy in this series. Authors distinguished two main groups of patients that require liver resection in IBDI: those with an injury-induced liver necrosis necessitating early intervention, and those in whom liver resection is indicated for treatment of liver atrophy following long-term cholangitis. In this commentary, indications for hepatectomy in patients with IBDI are discussed. Complex biliovascular injuries as indications for hepatectomy are presented. Short- and long-term results in patients fol-

lowing liver resection for IBDI are also discussed. Hepatectomy is not a standard procedure in surgical treatment of IBDI, but in some complex injuries it should be considered.

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**Key words:** Hepatectomy; Bile duct injury; Cholecystectomy; Laparoscopic cholecystectomy

**Core tip:** Different surgical biliary reconstructions are performed in most patients with iatrogenic bile duct injuries (IBDI). Roux-Y hepaticojejunostomy is the commonest biliary reconstruction. However, in some patients with complex IBDI involving disruption of hepatic confluence and injuries associated with concomitant vascular damage, hepatectomy is required. In this commentary, indications for hepatectomy in patients with IBDI are discussed. Complex biliovascular injuries as indications for hepatectomy are presented. Short- and long-term results in patients following liver resection for IBDI are also discussed. Hepatectomy is not a standard procedure in surgical treatment of IBDI, but in some complex injuries it should be considered.

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### COMMENTARY ON HOT TOPICS

I have read with great interest the recent article Li *et al*<sup>[1]</sup> analyzing the factors that had led to hepatectomy for patients with bile duct injuries after laparoscopic cholecystectomy (LC). I would strongly recommend this article to the readers.

Iatrogenic bile duct injuries (IBDI) are still a chal-

lenge for surgeons. The most frequently, they are caused by laparoscopic cholecystectomy which is one of the commonest surgical procedure in the world<sup>[2,3]</sup>. The early and proper diagnosis of IBDI is very important for surgeons and gastroenterologists, because unrecognized IBDI lead to serious complications such as biliary cirrhosis, hepatic failure and death<sup>[3,4]</sup>. Endoscopic techniques are recommended as initial treatment of IBDI. When endoscopic treatment is not effective, surgery is considered. Different surgical biliary reconstructions are performed in most patients in IBDI. Roux-Y hepaticojejunostomy is the commonest biliary reconstruction for IBDI. However, in some patients with complex IBDI, hepatectomy is required. Complex IBDI involve disruption of hepatic confluence and injuries associated with concomitant vascular damage<sup>[1,5]</sup>. The incidence of arterial injury in patients in IBDI ranges between 12% and 47%<sup>[6,7]</sup>. Distal IBDI are accompanied by damage of axial arteries (10%-15%) and proximal IBDI are usually associated with damage of the proper hepatic artery and its branches (most frequently right hepatic artery) (40%-60%)<sup>[2,6]</sup>. The incidence of complex biliovascular injuries has risen since the introduction of LC<sup>[8]</sup>. According to Li *et al*<sup>[1]</sup>, most complex IBDI such as Strasberg types E4 and E5 IBDI, either as an isolated condition or in combination with injury to vascular structures, can be effectively repaired by Roux-Y hepaticojejunostomy. However, in some patients with liver necrosis or lobar atrophy and fibrosis, hepatectomy is required<sup>[1]</sup>.

In their study, Li *et al*<sup>[1]</sup> analyzed the medical records of 76 patients who had received surgery for IBDI following LC from April 1998 to September 2007. Hepatectomy was performed in 10 of 76 patients (13.2%), with IBDI either as isolated damage or in combination with vascular injury (VI). Proximal IBDI (defined as disruption of the biliary confluence) and injury to the right hepatic artery were found to be independent risk factors of hepatectomy. When both injuries occurred, 72.7% (8/11) of their referred patients required hepatectomy. Five patients required early liver resection (within 5 wk post-LC) to control sepsis caused by confluent liver necrosis or bile duct necrosis. In five patients, hepatectomy was indicated during long-term follow-up (over 4 mo post-LC) to effectively manage recurrent cholangitis and liver atrophy. Based on their own observation, authors distinguished two groups of patients with IBDI that require hepatectomy: those with an injury-induced liver necrosis necessitating early intervention, and those in whom liver resection is indicated for treatment of liver atrophy following long-term cholangitis.

Another interesting aspect of this study is analysis of short- and long-term results in 10 patients undergoing hepatectomy. Authors noted high postoperative morbidity (60%) and mortality (10%), and satisfactory long-term results (with median follow-up of 34 mo) with either no or only transitory symptoms in 67% of the patients<sup>[1]</sup>.

There are a number of publications regarding the

use of hepatectomy in surgical treatment of complex IBDI<sup>[5,22]</sup>. Hepatectomy is one of therapeutic possibilities in patients with complex IBDI. Right hepatectomy is the most frequently performed liver resection in patients with IBDI because of the highest incidence injuries of the right hepatic artery<sup>[5,6,22]</sup>. Li *et al*<sup>[1]</sup> presented the following indications for hepatectomy in patients with IBDI: vascular injury causing liver necrosis without the possibility of vascular reconstruction, uncontrolled bile leakage due to a destructed segmental or sectional hepatic duct without the possibility of biliary reconstruction and recurrent cholangitis (more than four episodes) refractory to endoscopic management and not effectively amenable to bilioenteric anastomosis due to imaging evidence of atrophy or cirrhotic changes of the liver parenchyma. These indications are similar to literature data<sup>[5,22]</sup>. Most frequently, liver atrophy or necrosis, sepsis, and unreconstructable hepatic ducts, as complications of complex IBDI, and multiple failed previous repairs are indications for hepatectomy. Liver resection removes the fibrotic, atrophic segment and the diseased biliary confluence and allows good access to the remnant bile duct for a safe healthy anastomosis<sup>[5,6]</sup>.

Laurent *et al*<sup>[5]</sup> presented aims of hepatectomy in patients with complex IBDI involving biliary confluence (Bismuth IV, Strasberg E4). The aim of partial liver resection in patients with complex IBDI was to remove fibrotic and atrophic liver parenchyma with a high risk of secondary complications because of vascular or septic lesions. The other aim of hepatectomy was to remove completely the biliary stricture at the early stages of the disease for preventing progressive liver damage and potential malignancy caused by bile stasis and repeated cholangitis. Authors presented the following indications for hepatectomy: simultaneous ipsilateral portal and arterial injuries, stenosis of the hilar confluence involving secondary biliary confluence; presence of liver atrophy and presence of metallic stent.

Mercado *et al*<sup>[9]</sup>, based on their 20 years experience in surgical treatment of IBDI including 512 patients with complex IBDI (Strasberg E), performed major hepatectomy in patients with chronic biliary obstruction, liver atrophy, and persistent or recurrent cholangitis, with 1 to 3 previously failed attempts of surgical repair before arriving at their hospital. In remaining patients, Roux-Y hepaticojejunostomy without major liver resection was possible to perform. Authors pointed that in 2 patients, acute recurrent cholangitis, with pericholangitic abscess involving one hemi-liver that had not responded to medical and radiologic treatment, was the indication for major liver resection. In these cases, the liver parenchyma was not possible to rehabilitation, in spite of absence of disruption of hepatic influence. Based on the above mentioned two studies<sup>[8,9]</sup>, complex IBDI involving hepatic influence without vascular injury can be managed successfully with Roux-Y hepaticojejunostomy without major liver resection.

According to Thomson *et al*<sup>[10]</sup>, most patients are managed successfully with a Hepp-Couinaud hepaticojejunostomy because the left hepatic duct remains readily accessible. However, complications such as hepatic infarction, sepsis, anastomotic stricture, and intrahepatic stone formation can require hepatic resection, or even transplantation. Authors presented the following indications for hepatectomy in patients with IBDI: vascular injuries leading to partial liver devascularization, major injuries to the right hepatic duct that could not be repaired by conventional methods, and severe atrophy or sepsis of the hepatic lobe resulting from vascular injury or prolonged biliary obstruction that could not be drained effectively by Roux-Y hepaticojejunostomy. Liver transplantation was performed in combined biliary and vascular injuries leading to acute liver failure and in secondary biliary fibrosis with chronic liver failure.

In studies conducted in 1994 by Madariaga *et al*<sup>[14]</sup>, and in the year 1996 by Majno *et al*<sup>[15]</sup>, they indicated hepatectomy in cases of liver-infected necrosis. Sauvanet *et al*<sup>[16]</sup> described the following indications for hepatectomy: injuries from the confluence or higher with unilateral portal injury, right pedicle destruction, and liver atrophy. de Santibañes *et al*<sup>[21]</sup> presented algorithm for management of lobar atrophy including patients with IBDI. In asymptomatic cases of lobar atrophy, authors recommended control. Liver resection was indicated for symptomatic lobar atrophy caused by vascular injury, combined vascular and biliary injury, and biliary stenosis not responded to balloon dilatation. According to Truant *et al*<sup>[22]</sup> large review, the presence of a Strasberg type E4 or E5 BDI associated with hepatic artery injury was an independent risk factor for hepatectomy. Based on the analyzing studies in PubMed database authors presented the following indications for hepatectomy: recurrent biliary sepsis, biliary strictures caused by continuous cholangitis, intrahepatic abscesses, non-visualization and/or unsuitability of the proximal stump of the injured bile duct(s) for anastomosis, intrahepatic injuries of an aberrant right hepatic duct, anastomotic strictures and intrahepatic lithiasis, right hepatic lobar atrophy, secondary biliary cirrhosis, primary non-diagnosed Klatskin tumor.

It should be emphasized that immediate arterial reconstruction of biliovascular injuries recognized intraoperatively or at least within 4 d is recommended in order to avoid liver devascularization<sup>[8]</sup>. In another study, Li *et al*<sup>[11]</sup> presented hepatic rearterialization, with vascular reconstruction with or without vascular graft, when it was technically possible. Authors carried out hepatic resection only in patients with partial liver atrophy or necrosis. Only early recognition of vascular injury allows to perform rearterialization. In late recognized injuries, partial hepatectomy is required.

Partial hepatectomy is not only performed in patients with damaged liver parenchyma, such as liver ischemia, atrophy and necrosis. A minor partial hepatectomy is

used in order to improve biliary reconstruction in high intrahepatic IBDI. Mercado *et al*<sup>[23-27]</sup> described partial liver resection of segments IV and V that allowed adequate exposure of the bile duct at its bifurcation with an anterior approach of the ducts in order to perform a high quality anastomosis. The first partial-segment IV resection was performed in 1994.

The interesting phenomenon described by Li *et al*<sup>[11]</sup> was a higher incidence of vascular injuries, involving a right hepatic artery, in proximal IBDI compared to distal ones. In this series, 11/33 high injuries were associated with arterial injury but only 9/43 distal injuries were. Authors explained this phenomenon by the fact that the high biliary injury involving the confluence was more likely to damage the hilar component of the choledochal hilar plexus and thus prevent compensatory flow from the left artery. A high risk of concomitant vascular injury in patients with proximal IBDI has been also reported in other studies on LC-related complications<sup>[11,12,28-32]</sup>. Two arterial plexuses play important roles in adequate vascularization of the extrahepatic biliary system. One is the arterial plexus on the surface of the common bile duct and the common hepatic duct, connecting the posterosuperior pancreaticoduodenal artery and the right hepatic artery (most frequently, the 3 o'clock and 9 o'clock axial arteries). The other one (described by Vellar<sup>[33]</sup>) is located within hilar plate on the inferior surface of the hilum of the liver. It is formed by the collateral vessels coming from the right hepatic left hepatic arteries<sup>[33,34]</sup>. In patients with an occluded right hepatic artery combined with major bile duct injury, the arterial plexus on the bile duct is totally transected, and the hilar plate plexus might be jeopardized. In these cases, necrosis of the bile duct, dehiscence and stenosis of the biliary anastomosis, or a syndrome of multiple peripheral strictures within the biliary tree with jaundice and recurrent cholangitis, can occur<sup>[11]</sup>.

In the commented study, high morbidity and mortality rates and good long-term results were reported. It is associated with presence of serious complications in patients with complex IBDI requiring hepatectomy, such as peritonitis, sepsis with multi-organ insufficiency, and liver failure. Good long-term results show that liver resection should be considered in patients with complex IBDI that do not respond to other treatment. According to literature, hepatic resections in patients with IBDI can be performed successfully with low (0%) mortality, although with significant morbidity (50%-60%), and with excellent long-term success of 94%<sup>[6]</sup>.

In conclusion, depending on the time of surgical intervention, two groups of indications (early and late) can be distinguished. In the early postoperative period after cholecystectomy, hepatectomy is necessary in patients with liver necrosis or abscesses and bile leakage, in order to control peritonitis and sepsis. In remaining patients, hepatectomy is required in cases of recurrent cholangitis, that do not respond to standard therapy, and

symptomatic lobar atrophy. Although hepatectomy is not a standard procedure for patients with IBDI, it should be considered as a part of the surgical armamentarium for the repair of a selected group of patients in post-cholecystectomy injuries.

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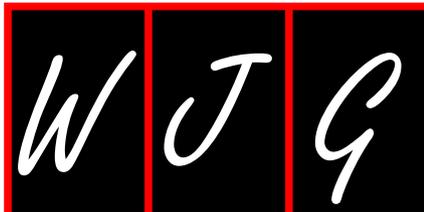
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WJG 20<sup>th</sup> Anniversary Special Issues (7): Liver transplant

## Right hepatic lobe living donation: A 12 years single Italian center experience

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### Abstract

Mini invasive techniques are taking over conventional open liver resections in the setting of left lateral segmentectomy for living liver donation, and hydride procedure are being implemented for the living related right hepatectomy. Our center routinely performs laparoscopic left lateral segmentectomy for pediatric recipient and has been the first in the Europe performing an entirely robotic right hepatectomy. Great emphasis is posed on living donor safety which is the first priority during the entire operation, then the most majority of our procedures are still conventional open right hepatectomy (RHLD), defined as removal of a portion of liver corresponding to Couinaud segments 5-8, in order

to obtain a graft for adult to adult living related liver transplant. During this 10 years period some changes, herein highlighted, have occurred to our surgical techniques. This study reports the largest Italian experience with RHLD, focused on surgical technique evolution over a 10 years period. Donor safety must be the first priority in right-lobe living-related donation: the categorization of complications of living donors, specially, after this "highly sensitive" procedure, reflects the need for prompt and detailed reports.

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**Key words:** Adult-to-adult living-related liver transplantation; Liver regeneration; Liver resections; Liver transplantation; Liver surgery

**Core tip:** A 12 years Italian single center experience is herein reported, focusing on the live donors who underwent conventional open right hepatectomies for adult to adult living related liver transplantations. In light of this experience we individualized three area of interest where we accomplished remarkable goals over this time period: donor nutritional status and rescue of steatotic donors; analysis of post hepatectomy liver regeneration; surgical technical developments.

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### INTRODUCTION

In July 2013 we have been communicated by the edito-

rial board of the *World Journal of Gastroenterology* that our paper “Analysis of Surgical and Perioperative Complications in Seventy-five Right Hepatectomies for Living Donor Liver Transplantation” published in May 2008<sup>[1]</sup>, had been cited, up to now, more than 27 times in the world English literature. This figure supported by data banks such as Scopus and ISI web of Science, made this scientific article entering in the 1% most cited papers of ever. Prompted by this achievement we reviewed our 12 years single Italian center experience with hepatic right lobe living donation. Indeed, we were particularly glad to contribute with this retrospective clinical report, to the special number of the *World Journal of Gastroenterology*, published to celebrate the 50<sup>th</sup> year’s anniversary of liver transplantation<sup>[2]</sup>.

Although in the last 6 years the number of procedures performed each year has been dramatically fallen down, our center is still, by far, the busiest living related liver transplant program in Italy (Table 1).

Herein, we will focus our attention on live donor of conventional right lobe (Coineaud segment 5-8) and only marginally on live donor of open left lateral segments (Coineaud segments 2-3)<sup>[3,4]</sup>. To further improve the outcome of these complex procedures, refinements in the surgical technique and better comprehension of the interrelations between post resectional liver regeneration, and donor nutritional status is, in our opinion, needed.

We individualized three area of interest where we accomplished remarkable goals over a 12 years period: nutritional status and rescue of steatotic donors<sup>[5]</sup>; analysis of liver regeneration in donors<sup>[1]</sup>; technical developments in conventional open resection for living donation<sup>[6]</sup>.

## PATIENTS POPULATION

At the “Istituto Mediterraneo per i Trapianti e Terapie ad Alta Specializzazione” in Palermo, Italy, from January 2002 to April 2013, we performed an overall of 107 live donor hepatectomy of which in details: 95 for adult patients and 12 for pediatric recipients. One additional case of potential right hepatectomy was aborted after the beginning of the surgery because of an abnormal venous outflow discovered at the intra-operative ultrasound and not previously detected at the pre operative imaging.

In the early phase of our experience two of the adult patients received a full left lobe (Coineaud segment 2-4), while the others adult recipients received a right lobe (Coineaud segments 5-8); in one case the right hepatic graft was harvested using a totally robotic procedure of retrieval. This case, the first performed in Europe and the second in the world, will not be treated here.

The pediatric recipients always received an anatomical left lateral segmentectomy (Coineaud segments 2, 3); in 8 cases we have performed entirely laparoscopic harvesting procedures and details of these procedures will not be discussed here<sup>[7]</sup>.

No living donor mortality, neither administration of heterologous blood transfusion were reported; 25 (27.1%)

**Table 1 Living liver donor potential candidates and type of surgical procedures performed at the Mediterranean Institute for Transplantation and Advanced Specialized Therapies, and the University of Pittsburgh Medical Center in Italy *n* (%)**

	Statistics
Exclusion criterion <sup>1</sup>	
Donor-related reasons	158 (44.0)
Donor withdrawal	43 (12.0)
Donor death	1 (0.3)
Exclusion at work-up:	
ABO incompatible	11 (3.1)
Psychology	18 (5.0)
Clinical/biochemistry	27 (7.5)
At imaging	
CT scan	30 (8.4)
MRCP scan	10 (2.8)
At liver biopsy	17 (4.7)
Other	1 (0.3)
Recipient-related reasons	93 (25.9)
Death awaiting LDLT	21 (5.8)
Drop-out (HCC progression)	21 (5.8)
LDLT refusal	12 (3.3)
Unsuitable to LDLT	10 (2.8)
Cadaveric OLT	29 (8.1)
Total excluded	251 (69.9)
Partial liver living graft <sup>2</sup>	
For adult recipients	
Full left lobe	2 (0.6)
Right lobe	94 (26.2)
For pediatric recipients	12 (3.3)
Left lateral segment	12 (3.3)
Total included	108 (30.1)
Overall	359 (100)

<sup>1</sup>Potential donors; <sup>2</sup>Suitable donors. CT: Computed tomography; MRCP: Magnetic resonance cholangiopancreatography; HCC: Hepatocellular carcinoma; LDLT: Living donor liver transplant; OLT: Orthotopic liver transplant.

living donors presented 1 or more episodes of complication in the post-operative period.

One case of HCV infection was revealed after donation<sup>[8,9]</sup>.

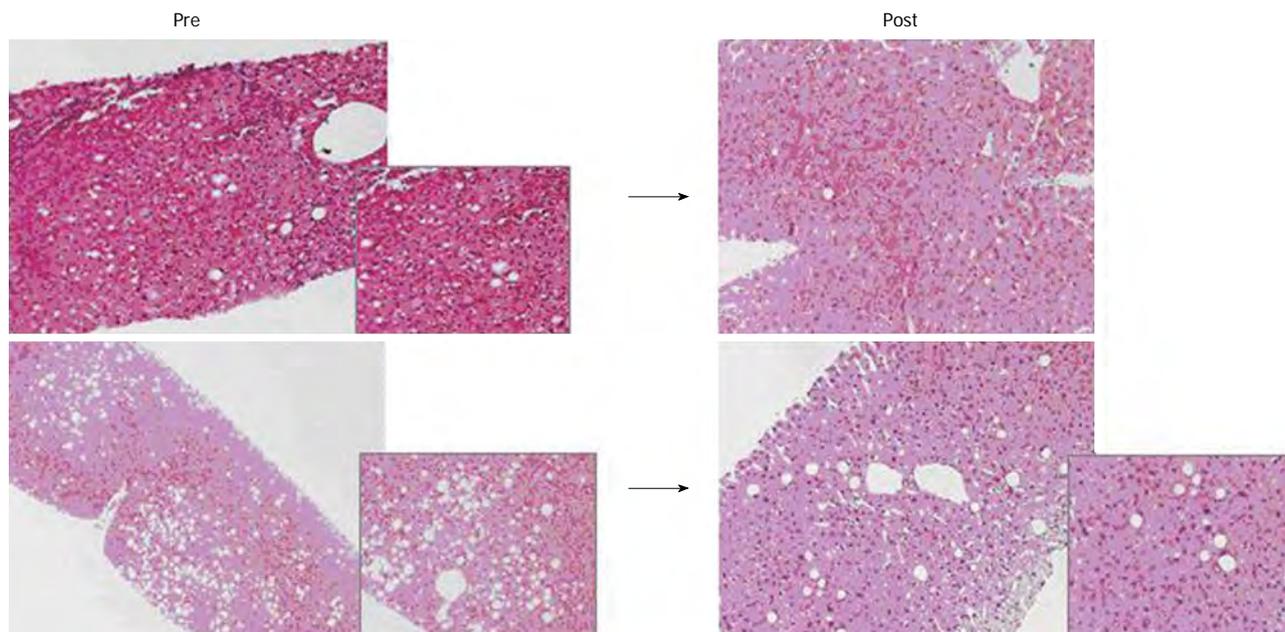
Regarding recipients, the patient and graft survival at 1, 3 and 5 year after right hepatectomy (RHLT) were 89.3%, 83.2%, 77.8% and 83.4%, 77.3%, 71.9% respectively.

## NUTRITIONAL STATUS AND RESCUE OF STEATOTIC DONORS

In our center a comprehensive step-by-step donor work-up protocol is designed to ensure donor and recipient safety.

Donors with hepatic steatosis > 30% are excluded from donation, due to reported impairment of both graft and patient survival after living donor liver transplantation (LDLT)<sup>[10]</sup>.

Donors with body mass index (BMI)  $\geq$  30 kg/m<sup>2</sup>, a significant correlation between BMI and overall grade of steatosis is well known<sup>[11]</sup> and/or steatosis at imaging



**Figure 1** Two histologic examinations of liver parenchyma with steatosis, before and after intensive dietary treatment in the same donor. 10/18 donors successfully completed the 3-mo treatment (body mass index < 30 kg/m<sup>2</sup>). In all 10 treated donors, hepatic macrosteatosis < 30% was found (range: 2%-15%) and therefore they were considered eligible for donation.

(ultrasonography, computed tomography or magnetic resonance imaging) or at histology underwent dietician consult and then re-evaluated within 3 mo.

After nutritional assessment [nutritional and dietary anamnesis, life-style evaluation and resting energy expenditure (REE) calculation] the dietician arranged a personal diet (carbohydrates 55%-57%, proteins 17%-19%, and lipids 24%-27%) and encouraged the donor to do physical activity.

Dietetic compliance of the donor was monthly reassessed.

Acceptable monthly weight loss was considered 2-4 kg, with a final gained BMI of < 30 kg/m<sup>2</sup>.

Eighteen potential living donors (age 27-59 years, male/female 14/4) were treated with diet. Nine out of 18 donors didn't complete the 3-mo dietary follow-up for donor-unrelated reasons. In 9 donors who successfully completed the 3-mo treatment a liver biopsy was performed. In all cases a hepatic steatosis degree < 30% was found and they became eligible as donors (Figure 1).

After LDLT, none of them experienced life-threatening complications or died. Liver function in both remnant liver donors and transplanted grafts showed a good outcome, with no differences (in terms of hospital length of stay, liver function parameter normalization after resection, and liver regeneration) between them and other LDLT without hepatic steatosis.

Surprisingly, in a very recent multicentric study, according to data maintained in the LiverMetSurvey database, a paradoxical survival advantage was observed in patients with steatosis undergoing liver resection for colorectal liver metastases (CLM)<sup>[12]</sup>.

This data has generated a fascinating hypothesis that

of excess body adiposity has a survival protective effect, concept which warrants further research.

## DONOR LIVER REGENERATION

Based on the evidence that an exposure of a small graft to persisting hyperdynamic circulation and high portal blood inflow may induce impairment of liver regeneration, and hepatic dysfunction<sup>[13,14]</sup>, we have translated in a population of 70 donors who underwent right hepatectomy the analysis of the impact of the donor regeneration predictors on post hepatectomy outcomes<sup>[1]</sup>.

Liver regeneration was evaluated with multidetector computed tomography (MDCT) at a mean of 61.07 d after surgery.

We have examined the possible impact of pre-surgical variables (*e.g.*, age, weight, height, BMI, liver function tests, creatinine levels, platelet counts, international normalized ratio, and glucose levels) and variables detected with preoperative MDCT imaging [*e.g.*, main portal vein diameter, steatosis, original liver volume, and spleen volume (SV)]. The future remnant liver volume (FRLV) was preoperatively calculated with a virtual surgical cut. Donor BMI was  $23.7 \pm 2.9$  kg/m<sup>2</sup>, and was used to physiologically assess nutritional status.

In 26 of the 70 donors analyzed (37.14%), 100% or greater hepatic regeneration had occurred at 2 mo.

There was no association between the clinical outcome and the liver regeneration rate. A stepwise multiple regression analysis showed that a higher BMI (coefficient = 0.035,  $P < 0.0001$ ) and preoperative parameters such as a smaller FRLV (coefficient = -0.002,  $P < 0.0001$ ) and a

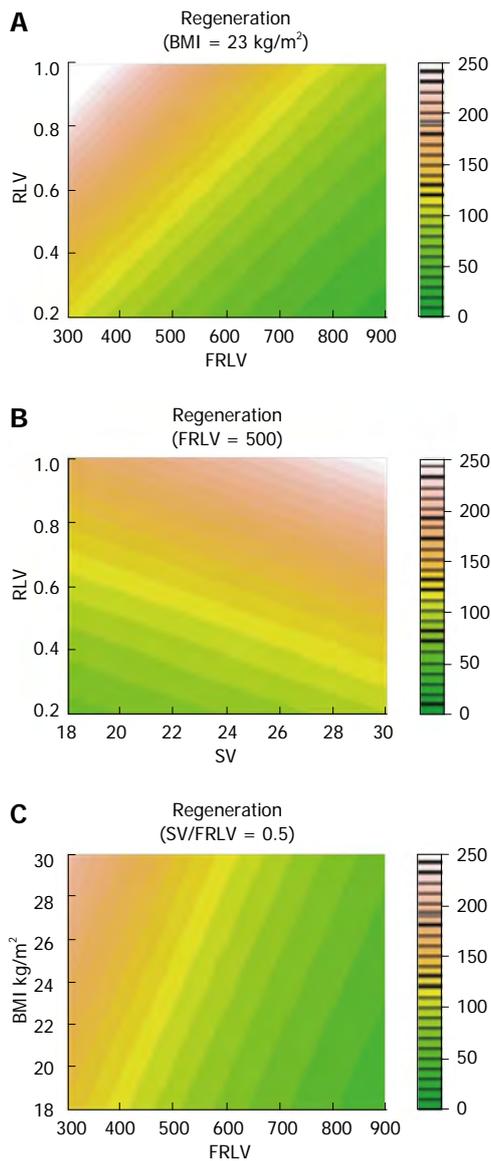


Figure 2 Locally Weighted Scatterplot Smoother graphics of the overall population, evidencing the association of percentage of liver regeneration with levels of body mass index (A), future remnant liver volume (B) and spleen volume/future remnant liver volume (C). FRLV: Future remnant liver volume; SV: Spleen volume; BMI: Body mass index.

greater SV/FRLV ratio (coefficient = 1.196,  $P < 0.0001$ ) were predictors of greater liver regeneration (Figure 2).

## TECHNICAL UPDATES

The most majority of our procedures are still conventional open RHLT, defined as removal of a portion of liver corresponding to Couinaud segments 5-8, in order to obtain a graft for adult to adult living related liver transplant.

While in the setting of the left lateral segment procurement open procedures are reserved only to rare cases presenting vascular anomalies.

During this 12 years period some changes, herein highlighted, have occurred to our surgical techniques. In

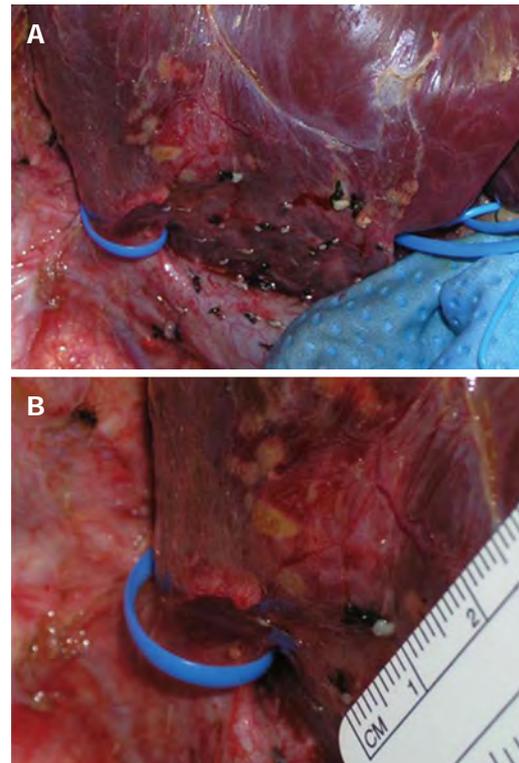


Figure 3 A large accessory hepatic vein draining the right lobe along with the right hepatic vein is encircled using a vessel loop (A). The measurement of the accessory hepatic vein [major of 5 mm, (B)] was performed for decision making to transect it with a vascular stapler, only when transections of liver parenchyma and of the structures of hepatic pedicle were almost complete.

particular we felt that the modifications we adopted concerning the transection of the accessories veins and final severing of the vascular stumps contributed to fasten, ameliorate and make safer the entire procedure.

Those technical development were possible using tools, strategies and experiences gained in laparoscopic surgery.

The operation is lately (after March 2008) being performed (last 10 cases, 9.2%) with a right renal flap incision while we were used to start with a bilateral subcostal incision, with upper midline extension (Mercedes incision). In the setting of the left lateral donation even an upper midline incision has been employed. For the right hepatectomy our original technique has been described elsewhere<sup>[15]</sup>.

We later adapted our technique to all type of anatomic variants. In the case of a right dominant hepatic vein, when a tributary of the hepatic venous system larger than 5 mm was encountered in the transection plane a test clamp was performed in order to see whether the liver parenchyma became dusky, after which a decision was made as to whether to preserve the branch.

This practice was lately substituted by the preoperative use of the MEVIS Hepavision<sup>®</sup> system in order to obtain a more detailed analysis of the outflow venous drainage.

In case of preservation we severed the accessory he-

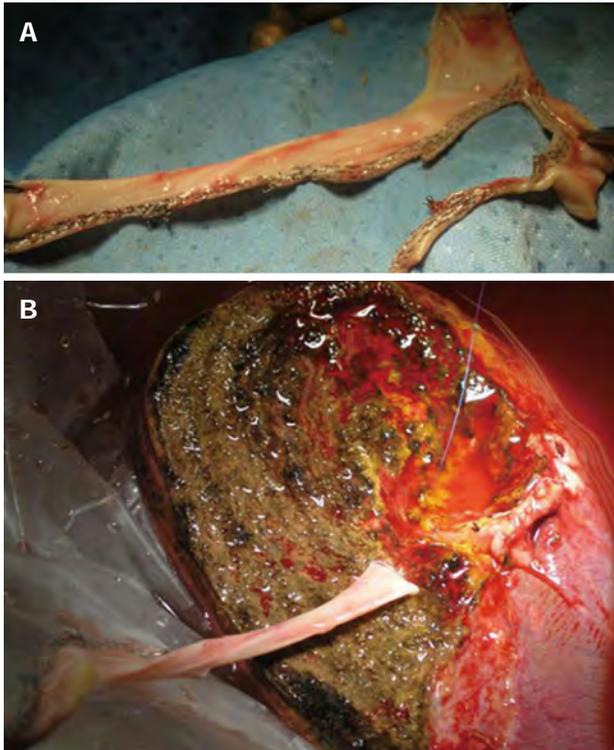


Figure 4 Usage of several vascular staplers were crucial to shapes and employees a heterologous venous conduit (A), previously harvested from a deceased donors, for a back-table reconstruction of a large accessory hepatic vein (B) to be connected to the recipient inferior vena cava in the LRLT.

patric vein, regardless was anterior or posterior, with the Endopath vascular staples (35 mm long, 12.3 mm wide; Ethicon Inc., Somerville, NJ) (Figure 3).

This step was originally taken using straight pediatric Pott vascular clamp and then suturing the two stumps of the vein.

This ultimate practice allowed us to avoid a complex manual suture especially in case of medial tributary when the parenchyma is not yet completely transected. Subsequently, once the parenchyma had been completely divided, the vascular stumps of the right branch of the portal vein, of the right hepatic vein and of the ipsilateral hepatic artery were sectioned with the Endopath vascular staples (35 mm long, 12.3 mm wide; Ethicon Inc., Somerville, NJ), while before the traditional Satinsky and Pott vascular clamps with subsequent manual suture were used.

In the setting of the left lateral segmentectomy, after mobilization of the left anatomic lobe and after performing the parenchymal transection as described above for the right counterpart, we lately substituted the conventional use of vascular Satinsky clamps with endo-vascular stapler.

In particular, while the vascular stumps of the left portal vein and of the left hepatic artery were treated with the Endopath vascular staples (35 mm long, 12.3 mm; Ethicon Inc., Somerville, NJ), the left or the common middle and left stumps of the hepatic veins was

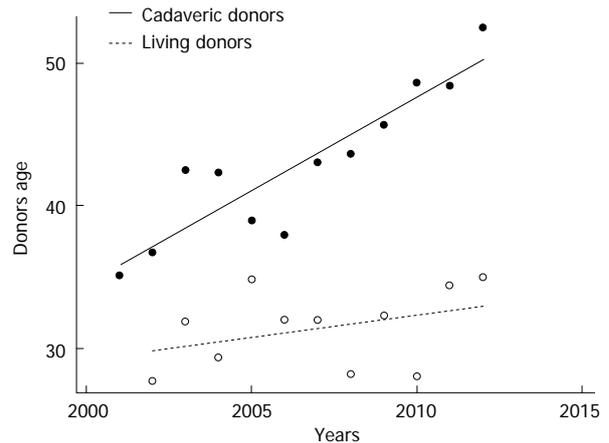


Figure 5 Linear regression models were used for explaining differences in terms of age between deceased and living liver donors over the last decade at the Mediterranean Institute for Transplantation and Advanced Specialized Therapies, and the University of Pittsburgh Medical Center in Italy. In our series of 677 cadaveric liver transplantation and 105 living donor liver transplantation, the deceased donor age has been increasing with a significant difference of average values between deceased and living liver donor age (95%CI: 0.9-1.3),  $P < 0.0001$ .

treated with Endo GIAT<sup>TM</sup> Universal 12 mm vascular stapler (Covidien, New Haven, CT).

Eventually we like to report on the utility of vascular endo-stapler into the open transplantation surgery, in the setting of the use of vascular graft. Indeed in few cases, the usage of several vascular staplers were crucial to shapes and employees a heterologous venous conduit, previously harvested from a deceased donors, for a back-table reconstruction of a large accessory hepatic vein which was connected to the recipient inferior vena cava of a LRLT (Figure 4).

## DISCUSSION

Deceased donor quality is worsening in Italy, the average of donor age in our center is much better in the living counterpart (Figure 5). However living donation carries a special risk and donor safety must be the first priority in liver living-related donation.

Indeed, the categorization of complications, the developments of new surgical tips, the changes matured over time in terms of donor selection and match needs to be promptly reported in details<sup>[16]</sup>.

Herein, we separately analyzed three area of interest in live donor hepatectomy that have been exposed to some changes and ameliorations since the beginning of our practice.

We found that the reduction of hepatic steatosis to a values of  $< 30\%$ , could be obtained with a strategic nutritional assessment and arranging an adequate personal diet. This concept is guiding our strategy in order to expand the live donor pool without affecting donor and recipient safety.

At this regard, the paradoxical survival advantage observed in patients with steatosis undergoing liver resec-

tion for CLM might create a new fascinating scenario, in which overweight living donor could be suitable for transplantation<sup>[12]</sup>.

However it is too early to conclude that peri-diagnosis overweight is a good prognosticator after a major liver resection, because its impact upon long-term survival is less well documented.

Further clinical studies with large series, comparing patients and potential live donors with different BMI, grade of liver steatosis and nutritional marker, will be necessary to obtain convincing evidence.

Assuming that preoperative nutritional status is one of the key points for successful resection in living-related liver donors, we have recently put emphasis not only on the evaluation of the ratio between donor and recipient liver volume but also on the predictors of optimal early liver regeneration in the donors.

Historical series suggested that in adult-to-adult living related liver transplantation one of the most challenging tasks is to match an optimal size graft, balancing the clinical condition of the sick recipient and the safety of the healthy donor. In this setting, particularly care must be taken in the pre operative imaging evaluation of liver and spleen volume<sup>[17-19]</sup>.

Eventually, in the scenario of the surgical refinements obtained over a 12 years period the adoption into the open conventional surgery of tools created for laparoscopic surgery such as the endo-mechanical stapler for vascular structures allowed us to make safer a unique surgical operation such as the living donor hepatectomy.

At this regard, we like to mention that traditionally one of the most stress full point of the operation is the positioning of the vascular clamp around the right hepatic vein or the common trunk of the middle and left hepatic vein and the subsequent manual suture of a potentially long vascular stump. Indeed, no matter how safely the clamp is placed the length of remnant vein to be sutured in the donor side might be short, and any moment the clamps could be displaced with detrimental consequences.

On the other hand, using the vascular stapler will make this step faster and safer with no consequence on the recipient side, once the stumps are opened on the back-table and the graft is flushed.

Adult-to-Adult Living-related Donor Liver Transplantation remains the greatest most recent and challenging evolution of liver transplantation, both from a technical and ethical point of view, which has contributed to reduce donor shortage<sup>[20]</sup>. Stringent criteria of donor selection criteria and peri-operative care were implemented following one of the first reported case of living donor death in 2002, and a smaller number of centers with large experience refined the surgical technique, selection and clinical management of both donors and recipients<sup>[21]</sup>.

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## Quality of care delivered to hospitalized inflammatory bowel disease patients

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**Key words:** Crohn's disease; Hospitalization; Inflammatory bowel disease; Quality improvement; Ulcerative colitis**Core tip:** Hospitalized patients with inflammatory bowel disease are at risk of harm and increased utilization of healthcare resources. Variation in the care delivered to these patients is common. There is room for improvement in the quality of care focusing on reducing admissions and identifying patients at risk for inpatient complications such as venous thromboembolism and *Clostridium difficile* infection. This review outlines several aspects of inpatient care in need of improvement and discusses a number of improvement strategies that have been implemented with potential to benefit both patients and providers.Weizman AV, Nguyen GC. Quality of care delivered to hospitalized inflammatory bowel disease patients. *World J Gastroenterol* 2013; 19(38): 6360-6366 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6360.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6360>

### Abstract

Hospitalized patients with inflammatory bowel disease (IBD) are at high risk for morbidity, mortality, and health care utilization costs. While the literature on trends in hospitalization rates for this disease is conflicting, there does appear to be significant variation in the delivery of care to this complex group, which may be a marker of suboptimal quality of care. There is a need for improvement in identifying patients at risk for hospitalization in an effort to reduce admissions. Moreover, appropriate screening for a number of hospital acquired complications such as venous thromboembolism and *Clostridium difficile* infection is suboptimal. This review discusses areas of inpatient care for IBD patients that are in need of improvement and outlines a number of potential quality improvement initiatives such as pay-for-performance models, quality improvement frameworks, and healthcare information technology.

### INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic gastrointestinal condition characterized by relapsing inflammation. Most patients with IBD are managed in the outpatient setting, however as disease severity progresses and complications arise, hospitalization is often required. Patients admitted to hospital are at increased risk for a variety of complications including venous thrombotic events (VTE), hospital acquired infections, *Clostridium difficile*, and death<sup>[1-3]</sup>. Moreover, hospitalized patients are more likely to require surgery<sup>[5,6]</sup>. There have been

conflicting reports on trends in hospitalization rates for IBD over the last decade and the literature has revealed significant variation in care and disease outcomes among hospitalized IBD patients. The heterogeneous nature of IBD severity, location, and phenotype as well as limited evidence to guide some therapeutic domains make standardization of IBD care delivery difficult. However, given that hospitalized patients are at the highest risk for morbidity, mortality, and health care utilization costs, quality improvement initiatives aimed at reducing variation, a known surrogate marker of poor performance, are well suited to this subset of patients<sup>[7,8]</sup>. This review outlines recent trends in rates of hospitalization for IBD and highlights areas of inpatient care that are in need of improvement.

## HOSPITALIZATION RATES FOR IBD

Most IBD care is delivered in the ambulatory setting. However, a significant proportion of patients will require hospitalization at some point in their disease course. Reports on overall trends in hospitalization rates for IBD over the past two decades are conflicting. Among a large cohort of patients followed across an integrated care network in Northern California, Herrinton *et al*<sup>[8]</sup> noted a 33% decline in hospitalization rates for Crohn's disease ( $P = 0.02$ ) and a 29% decline among those with ulcerative colitis ( $P = 0.0009$ ) from 1998-2005. However, a report using the National Hospital Discharge Survey (NHDS) showed that between the years 1970-2004, the rates of hospitalization for both Crohn's disease and ulcerative colitis in the United States increased<sup>[9]</sup>. Moreover, readmission is not uncommon, as demonstrated by Bernstein *et al*<sup>[10]</sup>, whereby 20% of patients with IBD were readmitted within the same calendar year. The most important advance in IBD care over the last ten years has been the increasing use of anti-tumour necrosis factor (TNF) therapy. The true impact of this on hospitalization rate may not have been completely captured in all these reports, thus more data is needed to evaluate the impact of anti-TNF on recent hospitalization trends.

While the literature on hospitalization rates is conflicting, most studies clearly show variation in practice patterns among hospitalized IBD patients. For example, in the cohort from Northern California discussed above, variability in surgery rates and immunomodulator use depending on the number of gastroenterologists and colorectal surgeons at each site was noted among the 16 medical centers included in the study<sup>[8]</sup>. Similarly, Spiegel *et al*<sup>[11]</sup> demonstrated significant variation among community and expert gastroenterologists in a number of care areas including patients admitted to hospital with severe ulcerative colitis. Expert gastroenterologists had a lower threshold to consult a surgeon for patients with severe steroid refractory disease. Outcomes following colectomy based on surgical volumes have also been shown in several studies, with high volumes centers having lower mortality rates<sup>[12,13]</sup>. Differences in outcomes based on the

type of admitting physician have also been demonstrated. Murthy *et al*<sup>[14]</sup> showed that patients with ulcerative colitis admitted to non-gastroenterologists had higher in-hospital mortality rates compared to those admitted under the care of a gastroenterologist (1.1% *vs* 0.2%,  $P < 0.0001$ ). Colectomy rates have also shown to be subject to geographic variation across the United States, with rates in the Midwest and West regions being three fold higher than those in the Northeast<sup>[15]</sup>. These studies underscore the need for improvement efforts focused on minimizing variation and bridging the gap between ideal and true performance in caring for the hospitalized inpatient with IBD.

## VENOUS THROMBOEMBOLISM PROPHYLAXIS

The risk of venous thromboembolism (VTE) has been shown to be increased among patients with IBD. Multiple studies have shown patients with IBD have a 2-3.5 fold increased risk for VTE compared to the general population and a recent meta-analysis confirmed a relative risk of 2.2 (95%CI: 1.83-2.65)<sup>[16-18]</sup>. In fact, one study showed that among 17 chronic illnesses, only heart failure and cancer carried a greater risk of VTE than IBD<sup>[19]</sup>. Moreover, it appears the prevalence of VTE among this group of patients is rising<sup>[1]</sup>. A number of risk factors for VTE among IBD patients have been identified. In a review of the Nationwide Inpatient Sample (NIS) between 1998-2004, Nguyen *et al*<sup>[11]</sup> identified increasing age, co-morbidities, ulcerative colitis (as opposed to Crohn's disease), surgery, and the need for public health assistance as important risk factors for the development of VTE. Disease activity has also been shown to be an important predictor, with one study showing a 4.5 fold increased risk of developing VTE during times of disease flare compared to remission<sup>[20]</sup>. Hospitalized IBD patients, particularly those with ulcerative colitis, appear to be at very high risk of VTE. Hospitalized IBD patients have been shown to have nearly a 6 fold increased absolute risk of VTE compared to an ambulatory IBD population<sup>[17]</sup> and an increased adjusted odds ratio of 1.85 (95%CI: 1.7-2.1) compared to those non-IBD patients admitted to hospital<sup>[11]</sup>. Moreover, VTE has been shown to be a marker of worse outcomes and higher health resource utilization. A review of a large database of hospital discharges in the United States found an odds ratio (OR) of 2.5 (95%CI: 1.83-3.43) for in-hospital mortality compared to IBD patients without VTE<sup>[1]</sup>. Mortality rates for ulcerative colitis were particularly high (37.4 per 1000 hospitalizations *vs* 9.9 per 1000 hospitalizations,  $P < 0.0001$ ). Patients with IBD and VTE also had a longer average length of stay (11.7 d *vs* 6.1 d,  $P < 0.0001$ ) and higher hospital charges compared to IBD patients without VTE.

Given the morbidity and mortality associated with inpatient VTE, the utility of VTE prophylaxis to prevent this complication is clear. Prophylaxis with heparin has been shown to significantly and safely decreased

the incidence of deep-vein thrombosis and pulmonary embolism<sup>[21]</sup>. However, despite the efficacy and ease of administering VTE prophylaxis, a significant percentage of IBD patients admitted to hospital are not receiving it and remain at risk. In a retrospective review of a tertiary IBD center in the United States, Tinsley *et al*<sup>[22]</sup> noted that the overall prophylaxis rate was only 67.6%. Variation was noted depending on the admitting service, with significantly higher rates noted among those admitted to a surgical service compared to a medical service (93.5% *vs* 57.4%). Even among those in which VTE prophylaxis was ordered, up to 34% of doses were not given. The lower prevalence for prophylaxis of IBD patients may in part be due to lack of awareness of their increased risk, as they are often young and mobile. This was suggested by a survey of gastroenterologists who were members of the American Gastroenterological Association<sup>[23]</sup>. Only 45% of respondents were aware that guidelines recommending VTE prophylaxis were published and a third surveyed reported working in a hospital with no protocols for VTE prophylaxis. Significant variation in practice was noted. However, contributors other than lack of awareness are suggested by studies of IBD experts. At a large Canadian tertiary IBD center, rates of VTE prophylaxis were lowest for patients admitted to the gastroenterology run IBD service compared to those admitted to general internal medicine or surgery<sup>[24]</sup>. Moreover, a survey of Canadian IBD experts found that almost 20% did not routinely use VTE prophylaxis and there was inconsistency among respondents regarding the indication for prophylaxis for patients in remission<sup>[25]</sup>. These studies underscore tremendous variation and suboptimal quality of care in preventing this morbid IBD related extra-intestinal manifestation. Given the uniform increased risk among hospitalized IBD patients, the presence of readily available and safe prophylactic agents, and the identification of important predictors for lack of prevention, this area of IBD care is a “low hanging fruit” that is very amenable to quality improvement initiatives.

## CLOSTRIDIUM DIFFICILE TESTING

A substantial body of evidence has emerged to implicate IBD as an important risk factor for *Clostridium difficile* infection (CDI). IBD patients have been shown to have higher infection rates with CDI compared to non-IBD patients. In an analysis of administrative data using a large registry of hospital discharges in the United States, Nguyen *et al*<sup>[4]</sup> noted that patients with ulcerative colitis (UC) had a prevalence of CDI that was 8 times that of non IBD patients admitted with a gastrointestinal problem (37.3 cases/1000 discharges *vs* 4.8 cases/1000 discharges,  $P < 0.001$ ). This finding was supported by a systematic review of 42 articles that showed CDI was more common among IBD patients than non IBD controls<sup>[26]</sup>. In addition to the higher prevalence of CDI among IBD patients, the incidence of CDI appears to be increasing over the last decade, particularly among hospitalized IBD patients. A review of discharges

among hospitalized IBD patients showed that the percentage of IBD admissions complicated by CDI had increased from 1.4% to 2.9% between the years 1998 and 2007 ( $P < 0.001$ )<sup>[27]</sup>. This increase was most marked for the subset with UC in which CDI complicated 5.3% of admissions. Similarly, in a retrospective review of hospitalized patients, Rodemann *et al*<sup>[28]</sup> showed that while CDI rates doubled among Crohn's disease patients between the years 1998 and 2004, they tripled among those with UC.

Not only does the literature support a true rise in CDI incidence and prevalence among individuals with IBD, but CDI also may confer worse outcomes. In-hospital mortality was four fold higher among IBD patients with CDI compared to those with IBD alone in a retrospective review of the NIS<sup>[27]</sup>. Similarly, a retrospective cohort study from Ontario, Canada showed a higher in-hospital mortality rate among hospitalized UC patients with CDI compared to those with UC alone (3.3% *vs* 0.38%,  $P < 0.0001$ )<sup>[29]</sup>. This increased mortality rate persisted out to five years of follow up in which the cumulative 5 years mortality rate was 27% for the CDI group and 14% for those with UC alone ( $P = 0.0073$ ). CDI has also been shown to increase length of stay and hospitalization costs among those with concomitant IBD. A review of a large administrative database of hospital discharges from the United Kingdom showed that median length of stay was 26 d among those with both CDI and IBD compared to only 5 d for those with IBD alone, a difference that was statically significant<sup>[30]</sup>. This translates into increased health care costs as shown by Nguyen *et al*<sup>[4]</sup>, whereby average hospital charges were \$35606 for a UC patient with CDI compared to \$23856 for those with UC alone ( $P < 0.0001$ ). The impact of CDI on colectomy is less clear. Jen *et al*<sup>[30]</sup> showed an increased risk of in-hospital colectomy among hospitalized UC patients with CDI as compared to UC alone (OR = 1.7, 95%CI: 1.4-2.1). This conflicts with the finding of Nguyen *et al*<sup>[4]</sup>, who showed a lower risk of colectomy in IBD patients with CDI (OR = 0.44, 95%CI: 0.34-0.55). Studies evaluating long term risk of colectomy after CDI are also conflicting. Navaneethan *et al*<sup>[31]</sup> showed that one year following hospitalization for UC, the colectomy rate was 35% for those with CDI during that hospitalization compared to 9.9% for those without infection ( $P < 0.001$ ). This was in keeping with a study from a large, tertiary IBD center in which one year colectomy rates for those with IBD and CDI were higher compared to those with IBD alone (44.6% *vs* 25%,  $P = 0.04$ )<sup>[32]</sup>. However, no difference in the risk of colectomy at 5 years was seen in the Canadian study cited above<sup>[29]</sup>.

The literature supports the finding that CDI among patients with IBD is a significant and increasingly prevalent problem, particularly for those with UC. Moreover, CDI confers increased short and long term mortality risk and increased health care utilization costs and may increase short and long term risk of colectomy. The majority of CDI is diagnosed within 48 h of admission, suggesting most patients acquire CDI in the community<sup>[28]</sup>. Given the high incidence and potential poor outcomes

associated with CDI and the fact that it is most often acquired before admission, routine testing of patients presenting with exacerbation of IBD for *Clostridium difficile* is a reasonable and potentially powerful intervention. In fact, a single center study showed a reduction in the number of colectomies after routine testing on admission was introduced<sup>[33]</sup>. While more evidence evaluating the benefits of routine testing is indicated, the literature thus far supports its use. Nonetheless, it appears routine testing is not widespread. A study of 34 European countries found tremendous variation in the incidence of CDI across hospitals and suggested difference in testing behavior was most likely responsible for these results<sup>[34]</sup>. Moreover, despite the rising prevalence of CDI, there is variation in approaches management in terms of antibiotic selection and practices regarding IBD specific immunosuppressive therapy. A survey of gastroenterologists in Canada and the United States found that nearly half of respondents add antibiotics to ongoing immunosuppressive therapy while the other half routinely held all immunosuppressants during antibiotic treatment<sup>[35]</sup>. The lack of consensus even among IBD experts highlights the need for more studies aimed at bringing clarity to the commonly encountered clinical “grey area”.

## INTERVENTIONS AIMED AT IMPROVEMENT

In order to adequately address gaps in care, an understanding of the contributing factors to the target problem is essential. It is important to tailor a quality improvement (QI) initiative to the local context and implement according to the resources, infrastructure, and QI culture available. A variety of methods to improve identified deficiencies in the quality of care of hospitalized IBD patients are already underway and discussed in detail below.

### Pay-for-performance program

Guidelines have outlined algorithmic approaches for following this complex group of patients. However, the uptake of IBD guidelines by gastroenterologists has been shown to be variable<sup>[36,37]</sup>. Therefore, other improvement approaches are necessary. A pay-for-performance (P4P) funding model has been advocated by some, whereby hospital and/or physician reimbursement is tied to meeting certain predetermined care benchmarks. This model is increasingly being used, although its impact on patient outcomes remains controversial. A review of over 7000 primary care physicians in the United Kingdom Quality and Outcomes Framework Pay for Performance Program found significant improvements in outcomes of a number of chronic diseases such as diabetes and coronary artery disease<sup>[38]</sup>. Similarly, a large study from the National Health Services in England compared mortality in a region of the country that had uniformly adopted a P4P model in all hospitals to the remainder of the country which did not use this model<sup>[39]</sup>. In the 24 hospitals that did use the P4P model, an absolute reduction in

**Table 1 American Gastroenterology Association Physician Quality Reporting System inflammatory bowel disease measures**

1	IBD type, location and activity all documented
2	Corticosteroid sparing therapy after 60 d
3	Bone loss assessment
4	Influenza immunization
5	Pneumococcal immunization
6	Testing for latent tuberculosis before initiating anti-TNF therapy
7	Assessment of Hepatitis B status before initiating anti-TNF therapy
8	Tobacco use: screening and cessation intervention

IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor.

mortality of 1.3% (95%CI: 0.4-2.1,  $P = 0.006$ ) and a relative reduction of 6% (95%CI: 260-1500) was observed. However, an American study evaluating the impact of the Centers for Medicare and Medicaid Services strategy that relies primarily on financial penalties through not providing hospitals with additional payment for health care-acquired or preventable complications found no significant changes in performance before or after this policy was adopted<sup>[40]</sup>. Therefore, while P4P programs hold promise, more study is needed before there is universal adoption of these models. Moreover, there is a need to evaluate the impact of these programs on IBD patient, given their complexity and unique needs. The American Gastroenterology Association has developed IBD specific quality indicators eligible for reimbursement through the Physician Quality Reporting System (PQRS) (Table 1)<sup>[41]</sup>. The impact of the PQRS on improving the quality of inpatient IBD care needs to be further characterized.

While not designed for the purposes of a reimbursement program, the Crohn's and Colitis Foundation of America have recently sponsored the publication of a set of quality indicators<sup>[42]</sup>. Both process and outcome indicators were developed that encompass a variety of domains in IBD care including treatment, surveillance, and health care maintenance. A number of inpatient IBD care process indicators are defined such as “IF a hospitalized patient with severe colitis is not improving on intravenous steroids within 3 d, THEN sigmoidoscopy with biopsy should be performed to exclude cytomegalovirus, AND surgical consultation should be obtained” as well as “IF a patient in whom a flare of IBD is suspected with new or worsening diarrhea THEN the patient should undergo *Clostridium difficile* testing at least once” and inpatient related outcomes measures including: (1) Number of days per year in the hospital attributable to IBD; and (2) Number of emergency room visits per year for IBD. It is important for gastroenterologists to become familiar with these quality indicators as they can be expected to become increasingly incorporated into the accreditation processes of health care institutions.

### Quality improvement frameworks

As the quality improvement movement continues to build momentum, there are increasing calls for innovative changes to the way health care is delivered. System rede-

sign is a fundamental principal in QI and there has been a particular focus on healthcare provided in the hospitalized setting as this is associated with significant morbidity and cost. Examples of new frameworks in IBD care are increasing. For example, a program in Australia implemented a new model of care consisting of a designated IBD service aimed at reducing hospitalizations<sup>[43]</sup>. The service consisted of a team of gastroenterologists, a designated weekly IBD clinic, a joint gastroenterology-surgery clinic, and a nurse practitioner (NP). The NP performed a variety of tasks including standardized protocols for monitoring patients on immunomodulator and biologic therapy, a 24-h help line, routine post-discharge follow up phone calls, and a routine education session at discharge. Outcomes were compared before and after adopting this framework. Following the implementation of the IBD service, the mean number of admissions per patient, mean length of stay, and total cost for inpatient care decreased. While this simple before and after design does not clearly control for biases, it does highlight the potentially valuable role of designated chronic care teams, particularly the role of the NP. NPs have been shown to improve outcomes in other chronic diseases, however their use in IBD has lagged behind other fields<sup>[44-46]</sup>. More studies are needed to evaluate their role in participating in IBD care.

Centralizing care delivery of certain disease into designated tertiary centers of excellence has also become a model employed by some jurisdictions. A number of large studies using administrative data have shown outcomes may be improved in high volume IBD referral centers. For example, United States hospital discharges were reviewed using the Nationwide Inpatient Sample between 1998-2004<sup>[6]</sup>. IBD patients admitted to high volume centers had lower in-hospital mortality compared to non-high volume hospitals. Similarly, Ananthakrishnan *et al*<sup>[13]</sup> found that patients admitted to high volume centers were more likely to undergo IBD surgery and had lower post-operative mortality rates compared to those in average volume hospitals. These studies support the designation of IBD centers of excellence whereby complicated IBD patients can be referred to for expert opinion and management. However, these centers must have the resources in place to handle such a complex cohort of patients and to be able to accommodate a large number of referrals to be seen in a timely fashion by gastroenterology and/or surgery.

### **Advancing healthcare information technology**

Hospitals have been increasingly incorporating healthcare information technology (HIT) into patient care. Many QI experts link HIT with improved quality, safety, efficiency, and coordination of care<sup>[47]</sup>. Hospitalized patients are at increased risk of harm in the form of hospital acquired infections, preventable complications (*e.g.*, VTE), medication errors, and lapses in communication at discharge regarding follow-up. Therefore, initiatives aimed at reducing these harms are needed, and HIT is one avenue that may

achieve improvements. If designed well and appropriately adapted to the context of a given institution, an electronic health record has the potential to improve efficiency, safety, and communication. Computerized provider order entry has the potential to decrease medication errors, link providers to clinical decision support, and address the underuse or overuse of certain resources<sup>[47]</sup>. For example, standardized admission order sets involve a collection of orders or investigations that when designed well, are effective through improving efficiency, decreasing variation, enhancing workflow, and improving communication of evidence based practices<sup>[48,49]</sup>. Fields can be customized to an admitting service (*e.g.*, general surgery, gastroenterology, *etc.*) or disease specific (*e.g.*, IBD). An IBD admission order set has the potential to address areas in which the quality of care is suboptimal. For example, including *Clostridium difficile* testing on the admission order may be expected to increase the rates of screening for IBD patients presenting to hospital with new or worsening diarrhea. While the impact of such initiative on IBD outcomes is not yet known, it would increase adherence to recently defined QI benchmarks and potentially identify a high risk group for bad outcomes<sup>[42]</sup>. Similarly, an electronic order set that automatically defaults to ordering VTE prophylaxis on admission may improve the underuse of VTE prophylaxis outlined above. The physician would deliberately have to remove this order if it is not desired. These “forcing functions” are regarded among the most effective patient safety interventions available<sup>[50]</sup>. This strategy has been shown to be effective in increasing prophylaxis rates in several studies of non-IBD patients and overcomes barriers to ordering VTE prophylaxis such as the knowledge gaps outlined above<sup>[51,52]</sup>. However, other barriers to VTE prophylaxis have also been identified that may not be adequately addressed by an order set. Moreover, evidence in support of VTE order sets in IBD is lacking. This underscores the importance of a clear understanding of the local context before implementing an initiative and to ensure that it is well tailored to the patients, resources, and providers at a given institution. Nonetheless, the theory behind order set effectiveness is sound and more study is needed to evaluate their impact on IBD outcomes.

## **CONCLUSION**

In summary, hospitalized patients with inflammatory bowel disease are at risk of harm and increased healthcare utilization resources. More attention needs to be placed on reducing hospital admissions and re-admissions and preventable inpatient complications such as VTE. A number of potential improvement strategies may benefit both patients and providers including pay-for-performance programs, quality improvement frameworks, nurse practitioners, and healthcare information technology. While the true impact of these interventions on IBD outcomes still needs to be elucidated, quality indicators are expected to become increasingly measured in all aspects

of clinical care and it is therefore important that IBD providers familiarize themselves with these concepts.

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## Quality improvement in pediatric inflammatory bowel disease: Moving forward to improve outcomes

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### Abstract

In recent years, pediatric health care has embraced the concept of quality improvement to improve patient outcomes. As quality improvement efforts are implemented, network collaboration (where multiple centers and practices implement standardized programs) is a popular option. In a collaborative network, improvement in the conduct of structural, process and outcome quality measures can lead to improvements in overall health, and benchmarks can be used to assess and compare progress. In this review article, we provided an overview of the quality improvement movement and the role of quality indicators in this movement. We reviewed current quality improvement efforts in pediatric inflammatory bowel disease

(IBD), as well as other pediatric chronic illnesses. We discussed the need to standardize the development of quality indicators used in quality improvement networks to assess medical care, and the validation techniques which can be used to ensure that process indicators result in improved outcomes of clinical significance. We aimed to assess current quality improvement efforts in pediatric IBD and other diseases, such as childhood asthma, childhood arthritis, and neonatal health. By doing so, we hope to learn from their successes and failures and to move the field forward for future improvements in the care provided to children with IBD.

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**Key words:** Inflammatory bowel disease; Colitis; Ulcerative; Crohn's disease; Child; Adolescent; Quality of health care; Review

**Core tip:** This review article provides an overview of the quality improvement movement and the role of quality indicators. Active quality improvement efforts in pediatric inflammatory bowel disease are discussed, and the need for standardizing the development of quality indicators across all fields of healthcare is emphasized. This article also discusses the importance of incorporating validation techniques when developing and selecting quality indicators. Examples of quality improvement efforts in other areas of pediatric chronic illnesses are presented, with important lessons highlighted to guide future quality improvement initiatives.

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## INTRODUCTION

The inflammatory bowel disease (IBD) are a group of chronic gastrointestinal diseases caused by inflammation of the gastrointestinal tract and resulting in malabsorption of nutrients, failure to thrive, abdominal pain, and extraintestinal manifestations<sup>[1]</sup>. They consist of two main subtypes: Crohn's disease (CD) and ulcerative colitis (UC), and patients who do not fall into either subtype are deemed IBD type unclassified (IBD-U)<sup>[1]</sup>. Adult and pediatric onsets of IBD differ in some regards, with one of them being in regards to the degree of psychosocial burden. Quality of life is significantly affected, with children being frequently affected by psychosocial issues as a result of stunted growth, weight gain from drug therapy and the inability to feel confident around peers due to associated bowel issues<sup>[2]</sup>.

Incidence and prevalence of pediatric IBD have been increasing worldwide. A recent systematic review with the aim of describing international trends for pediatric IBD rates found that 60% and 20% of relevant publications reported statistically significant increases in CD and UC incidence, respectively<sup>[3]</sup>. The findings represented data from 32 countries, thereby providing evidence that pediatric IBD has become a global disease affecting a multitude of countries<sup>[3]</sup>. Several developed countries had released reports characterizing incidence rates within their pediatric population. In Ontario, Canada, there was a 5% and 7.6% increase per year in incidence for children aged 0-4 years and 5-9 years, respectively<sup>[4]</sup>. Similar increases have been demonstrated in Spain and Northern California, United States<sup>[5,6]</sup>.

With increasing incidence and prevalence comes greater economic burden, both on the healthcare system and on patients' families. Based on 2003-2004 data, the direct healthcare costs of IBD in the United States was \$3.1 billion for CD and \$2.1 billion for UC<sup>[7]</sup>. Children had the highest cost of direct medical care, and lengths of hospital stay were also high, with an average of 8.1 d for CD patients who were  $\geq 5$  years of age<sup>[8]</sup>. While the average pediatric patient with IBD costs significantly more in direct medical costs than the average adult, a high degree of variability in care and outcomes has been noted in the literature<sup>[7]</sup>. A study from the United States demonstrated variation in care provided to children in a network of pediatric IBD centers, including a large degree of variation in use of immunosuppressive medications at diagnosis<sup>[9]</sup>. Similarly, we have previously described variation in surgical outcomes in Canadian children based on family income, despite a universal access healthcare environment<sup>[10]</sup>. In addition, we described a high degree of variability in medication prescription rates in children with IBD from three countries<sup>[11]</sup>. This variation in care may be unwarranted, and indicate room for improvement in the quality of care<sup>[12]</sup>. The description of unwarranted variation in care has therefore spurred quality improvement efforts in pediatric IBD<sup>[13]</sup>.

In addition, with increasing burden of pediatric IBD,

the issue of quality of care becomes more important. Improved quality of care should lead to improved outcomes, and therefore lower long-term burden as well as medical and psychosocial benefits. While the motivation for improving processes involved in providing high quality medical care is clear, such quality improvement efforts should be based in evidence and undergo validation to ensure efficient resource allocation.

Recognizing the disparities present in modern-day healthcare systems, the Institute of Medicine released two reports highlighting current issues affecting quality<sup>[14,15]</sup>. Both reports have argued that quality of care is sub-optimal across all aspects of health regardless of disease type, and have proposed that healthcare systems be reformed to prevent mis-use of healthcare services<sup>[16]</sup>. As a result of these reports, many providers, including pediatric IBD specialists, have worked with quality improvement experts to improve the quality of care for their patients by implementing quality improvement programs. We have reviewed current published quality improvement efforts in pediatric IBD, and the evidence that they have improved outcomes. In addition, we have examined evidence from quality improvement work in other fields to inform future pediatric IBD efforts and improved their likelihood of success.

## WHAT IS QUALITY IMPROVEMENT?

Quality improvement in medicine is defined as the effort to change care using an evidence-based approach in order to make tangible positive changes to the delivery healthcare<sup>[17]</sup>. With origins stemming from the field of industry and production, quality improvement efforts have slowly been introduced into the field of healthcare delivery over the past few decades. Definitions used to describe common quality improvement terms can be found in Table 1.

The plan-do-study-act (PDSA) has been used as a paradigm for quality improvement efforts<sup>[18,19]</sup>. With this framework, at the plan stage, quality indicators are developed to measure the quality of care provided. During the do stage, these indicators are implemented into practice and quantitative measures are collected. At the study stage, the statistics gathered in the previous stage are used to evaluate the progress that this action has on healthcare delivery. At the act stage, a feed-back loop is utilized such that quality indicators which have produced sub-optimal results are re-examined and cycled back through the PDSA cycle. Quality indicators which have improved quality of care are also re-examined to ensure that additional modifications cannot be added to ensure optimal care is being provided<sup>[20]</sup>. The National Health Services(NHS) in the United Kingdom has recommended that the PDSA be used in trial phase and then implemented fully once outcomes have been satisfied<sup>[20]</sup>. A modified PDSA cycle can be found in Figure 1, and is adapted from Langley *et al*<sup>[19]</sup>.

In general, many efforts for quality improvement have been unsuccessful due to the lack of a trial phase,

**Table 1** Definitions of common quality improvement terms<sup>[17,18,22,23]</sup>

Term	Definition
Quality improvement	The overall framework used to describe the process of implementing evidence-based interventions to bridge the disparities currently present in various healthcare systems
Quality indicators	A set of measures used to assess the appropriateness and quality of health care. Quality indicators are considered the fundamental building blocks of quality improvement efforts
Structural indicators	Indicators having to do with the structure of the healthcare system ( <i>e.g.</i> , staffing, equipment, environment, electronic health records)
Process indicators	Indicators having to do with the process of providing care ( <i>e.g.</i> , investigations, treatments, interaction with patients)
Outcome indicators	Indicators having to do with assessing the outcome of patients ( <i>e.g.</i> , mortality, morbidity, quality of life, patient satisfaction)

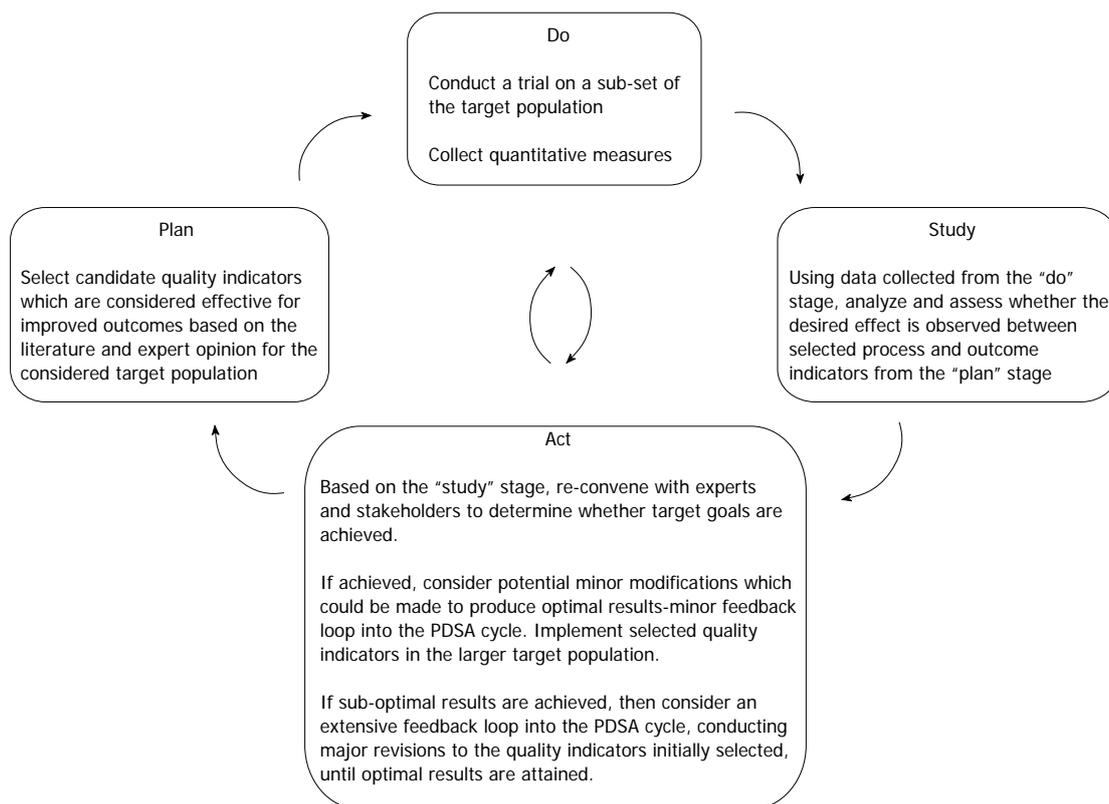


Figure 1 Modified plan-do-study-act cycle. PDSA: Plan-do-study-act.

and the lack of feed-back and change. Too often, quality indicators are developed and measures are extracted, but the process does not extend further beyond that point<sup>[21]</sup>.

### WHAT ARE QUALITY INDICATORS?

The process of quality improvement of medical care requires markers of adequate and inadequate care. The essential building blocks for quality improvement efforts are the proper identification and implementation of effective quality indicators<sup>[22]</sup>. These quality indicators are measurable elements of practice performance for which there is evidence or consensus that they may be applied to assess and improve the quality provided<sup>[23]</sup>. The types of quality indicators have been broadly categorized as follows: (1) Structural measures-[indicators to do with the structure of the health system (*e.g.*, staffing, equipment, electronic medical records)]; (2) Process measures-[indicators to do with the process of providing care (*e.g.*,

investigations, treatment, interactions with patients)]; and (3) Outcomes measures-[indicators which assess the outcome of patients (*e.g.*, mortality, morbidity, quality of life, patient satisfaction)]<sup>[18]</sup>. While improvement in all categories of indicators is desirable, process measures have garnered the majority of the attention, as they are most easily modified. To serve their intended purpose, process measures should predict facility-level outcomes, predict patient-level outcomes, and specify changes in care that are supported by the scientific evidence while being acceptable to patients and clinical staff<sup>[24]</sup>.

### QUALITY IMPROVEMENT IN PEDIATRIC IBD

Understanding the benefits associated with standardized quality improvement efforts, an initiative called ImproveCareNow (ICN) was implemented amongst several

centers in the United States, and is rapidly expanding<sup>[25]</sup>. It consists of a network of IBD centers engaged in a well-designed quality improvement program with an overall aim to determine whether measuring and decreasing variability would improve remission rates and other outcomes<sup>[25]</sup>. Patient details and center practices are inputted prospectively into a registry, with quality indicator compliance rates fed back to centers on a regular basis. This feedback mechanism forms the basis of well-planned quality improvement efforts, including comparative reports, knowledge sharing activities, and clinical pre-visit planning mechanisms. The participating centers can then use their own results as benchmarks and compare future results as markers of improvement. They can also compare their performance to other participating centers<sup>[26]</sup>. Initial results from ICN activities are promising, with improved compliance and remission rates demonstrated in the earliest years of the program. Crandall *et al*<sup>[27]</sup> reported improvement in adherence to the selected quality indicators based on prospectively collected data from 6 participating centers. This was associated with a higher proportion of patients with inactive disease by Physician Global Assessment (PGA). However, improvements were relatively modest (13% improvement in remission rates for CD, 11% improvement for UC, based on statistical process control methods). These improvements were associated with a decreased proportion of patients with mild active disease. The proportion of patients with moderate to severely active disease remained stable over time. In addition, improvements in remission rates measured by the more objective short Pediatric Crohn's Disease Activity Index (sPCDAI) were smaller than those measured with PGA<sup>[13,28]</sup>. This raises the issue of disease activity measurement in IBD. As evidence grows that clinical remission is insufficient to predict long-term prognosis, the use of measures which correlate strongly with mucosal healing and complete remission becomes especially important<sup>[29]</sup>.

In another study, Cincinnati Children's Medical Centre, one of the original participating centers in ICN with a long history of quality improvement efforts, published preliminary results of their quality improvement program in a separate report<sup>[30]</sup>. As with ICN, a registry was developed, and indicators and outcomes were measured. To assess remission rates, PGA was used, along with patient-reported symptoms. Other variables measured included use of azathioprine and corticosteroids. They also assessed the use of vitamin D supplementations and serum 25-hydroxyvitamin D levels. Process and outcome indicators were chosen based on available guidelines and expert consultation. The institution reported improved remission rates of 59% to 76%, ( $P < 0.05$ ), and a decreased use of repeated steroid courses of 17% to 10%, ( $P < 0.05$ ). Investigators also found significant associations between decreased disease activity and vitamin D supplementations as well as disease activity and serum 25-hydroxyvitamin D levels ( $P = 0.02$ ), although there was no control for confounders such as overall medica-

tion adherence and frequency of clinic visits<sup>[30]</sup>.

While ICN has become the first large-scale pediatric IBD quality improvement network to demonstrate successful changes in practice, some lessons can be learned from their methods (as well as those of quality improvement efforts in other pediatric patient groups) to further increase the likelihood of success in future quality improvement efforts.

## QUALITY INDICATOR DEVELOPMENT AND VALIDATION

The indicators developed by ICN formed the basis of the measurement and feedback system, and therefore were developed with the assumption that improvement in the care provided and outcomes achieved would follow improved compliance with these indicators.

The initial set of indicators developed by ICN were not considered adequate and were revised<sup>[25]</sup>. The initial 19 measures initially deemed appropriate for improving pediatric IBD quality were implemented amongst multiple centers. As these measures were being used in routine practice, it became obvious that several quality indicators needed further clarification, and some measures were not appropriate or feasible for inclusion<sup>[25]</sup>. Flexibility is therefore required in the development and implementation of a quality improvement network, and the allowance for revision is an important part of the quality improvement process.

A pilot phase, as conducted by ICN is also important to ensure that intervention in the population being studied will produce a desirable effect. While quality indicators in quality improvement efforts are typically derived using RAND appropriateness methodology, which integrates expert opinion and review of the evidence, the literature may not be representative of the centers involved in the network<sup>[24]</sup>. For example, a quality improvement network could consist of centers whose patients are mostly from low income neighborhoods. Measurement and control for these confounding factors is paramount. Without a pilot phase, and assessment of confounding, a formal quality improvement network may use imprecise process measures, leading to wasted resources and possibly misleading information<sup>[24]</sup>. Following development of a second set of indicators for ICN, various mechanisms were put into place to provide clarification (such as a manual detailing strategies for accurate and complete measurement by participating centers). Of the 19 quality indicators developed, the quality indicators assessed by Crandall *et al*<sup>[25,27]</sup>, through ICN can be found in Table 2.

Both sets of ICN quality indicators were developed using RAND appropriateness methodology. Briefly, experts convene twice, before and after a meeting to rate importance of items derived from existing medical literature<sup>[31]</sup>. Median scores are calculated and a final list is developed<sup>[32]</sup>. Although reliability, feasibility and validity of indicators using the RAND appropriateness method have been established, improvement in the performance of

**Table 2** ImproveCareNow quality indicators assessed in Crandall *et al*<sup>[25,27]</sup> (of 19 total indicators developed)

Original set of quality indicators	Modified set of quality indicators	Results of quality improvement
Process: Diagnostic evaluation, disease phenotype, disease severity, body mass index including height and weight are all presented as separate measures under the domain titled: "Initial Diagnostic Evaluation"	Process: Assessing disease phenotype, disease severity, body mass index including height and weight were combined into a single "bundled" domain titled: Model classification	Increase in complete disease classification through the "bundled" measures: CD 38% <sup>b</sup> increase, UC 27% <sup>b</sup> increase
Outcome: Nutritional and growth status (those "at risk" with evaluation plans and those currently experiencing "failure" with treatment plans) are presented as separate domains	Outcome: Nutritional and growth status (those "at risk" and those currently experiencing "failure") are combined into the same domain, with no reference to further intervention plans based on the assessed status	Nutritional status: No changes in BMI z-scores for CD, however there was a 0.11 decrease in BMI z-score for UC ( $P = 0.01$ ) Growth status did not change for CD and UC
Process: Treatment measures listed consist of measuring TPMT levels to ensure appropriate doses of thiopurine are prescribed	Process: Several other treatment quality indicators were included under the domain titled Treatment Measures which were not included in the original set such as anti-TNF therapy, skin test, screening for tuberculosis, appropriate infliximab and methotrexate dosage, among several others  Outcome: Remission as an outcome measure was added (overall remission, prednisone free remission and sustained remission) The absence of prescribing prednisone was also an added outcome measure	Improved compliance with TPMT status assessment before prescribing thiopurines: CD 20% <sup>b</sup> increase, UC 23% increase Improvement in appropriate dose: CD 8% <sup>b</sup> increase, UC 41% <sup>b</sup> increase  Only those with mild disease had significant changes to disease activity for CD and UC Remission rate (sPCDAI) increased 4% ( $P < 0.0001$ ) Proportion with inactive disease improved: CD 13%, UC 11% Proportion who were not on prednisone increased by 4% for CD

<sup>b</sup> $P < 0.01$ . CD: Crohn's disease; UC: Ulcerative colitis; BMI: Body mass index; TPMT: thiopurine methyltransferase; TNF: Tumor necrosis factor; sPCDAI: Short pediatric Crohn's disease activity index.

the selected indicators do not necessarily correlate with improved outcomes<sup>[33]</sup>.

The typical indicator development process does not include a validation stage to ensure that the effects on outcomes are desirable. An alternative to the RAND appropriateness method incorporating a validation stage was proposed by Harris *et al*<sup>[24]</sup> in the context of an alcohol addiction program. First, outcomes were collected and compared from pre- and post-treatment in a large sample of the target population. The goal of this stage was to determine whether implementing an effort to improve the completion of selected quality indicators would improve scores from baseline<sup>[24]</sup>. A candidate set of quality indicators were selected from available literature, and association between selected indicators and outcomes were evaluated, using statistical methods and controlling for important confounding variables. As several predictors were tested for effects, true positives were maximized and false positives were minimized to avoid detection of spurious associations<sup>[24]</sup>. Finally, those indicators which demonstrated the highest statistical correlation with outcomes were cross-validated with another subset of patients from the target population to determine whether the effect is sustained. Lastly, expert consultation was re-convened and indicators were re-evaluated<sup>[24]</sup>. This approach may result in indicators that are more closely correlated with outcomes, thereby maximizing the cost-benefit ratio of implementing a formal quality improvement network.

Ideally process measures which indicate quality should be associated with both facility-level and patient-outcomes<sup>[24]</sup>. Outcomes chosen by ICN as important

measures of success include remission rates (as measured by both PGA and PCDAI), nutritional status [measured by body mass index (BMI) z-score], linear growth velocity, and steroid-free treatment rates<sup>[27]</sup>. Some of the indicators chosen would not directly correlate with these outcomes. For example, thiopurine methyltransferase (TPMT) genotype status would dictate safety of use of azathioprine or 6-mercaptopurine and risk of adverse events, but may not directly affect remission or growth velocity. In addition, completion of TPMT genotype is restricted to certain regions with some centers preferring TPMT phenotypic expression testing, and others preferring to monitor complete blood count and/or serum azathioprine metabolite levels. Therefore, TPMT genotype measurement may predict avoidance of serious adverse events, but may not be associated with either patient-level or facility-level outcomes<sup>[34]</sup>.

In summary, while ICN has successfully demonstrated improved documentation and compliance with select indicators, only modest benefits in patient outcomes have been achieved. Rigorous pilot work, with assessment and validation of correlation between indicators and outcomes could improve success. Elimination of indicators that are unassociated with outcomes would reduce the burden on participating centers and improve the cost benefit balance of a quality improvement network.

## LESSONS LEARNED IN OTHER PEDIATRIC QUALITY IMPROVEMENT PROGRAMS

As the idea of quality improvement in health care has

become increasingly significant, several network collaboratives have been created with the overall goal of improving child health. A recent review by Billett *et al*<sup>[35]</sup> highlighted five well-established and impactful regional and national pediatric quality improvement networks in the United States. The networks were in the fields of IBD (ICN), childhood asthma care, perinatal care, patient safety, and central line associated blood stream infection prevention in intensive care patients.

Although the review identified five examples of successful collaboratives, there are many other collaboratives in existence which have been able to demonstrate successes in their endeavors as well. The Canadian Neonatal Network (CNN) is a large network which includes upwards of 30 neonatal centers across Canada, with the goal of improving care in intensive care units, and therefore improving neonatal outcomes. Information on patients are collected in a database, which is then subsequently used to inform selection of indicators and to benchmark progress. Quality improvement is a priority of the CNN, as demonstrated by the creation of evidence-based practice for improving quality (EPIQ) cluster randomized controlled trial<sup>[36]</sup>. EPIQ aimed to reduce nosocomial infections and bronchopulmonary dysplasia (BPD). Results demonstrated significantly reduced nosocomial infections and BPD in the quality improvement intervention group compared with control centers<sup>[36]</sup>. In EPIQ, evidence based literature is used to inform the selection of quality indicators and information collected from the database is used to inform the use of the most appropriate indicators<sup>[37]</sup>. Based on the EPIQ trial to assess the association between indicators and outcomes, the collaborative is now confident that these indicators can be used to determine high quality care in all centers involved in the CNN.

Another pediatric collaborative network aimed to improve the quality of care received by children with asthma presenting to emergency departments<sup>[38]</sup>. Process and outcome quality indicators were chosen from an existing adult quality initiative, where associations between the selected process indicators and outcomes were observed<sup>[39]</sup>. Unfortunately, preliminary results from the pediatric collaborative did not find an association between these process indicators and outcomes, indicating the importance of validation of indicators in the specific patient group to which they will be applied prior to their widespread application<sup>[38]</sup>.

Another quality improvement network in pediatric asthma based their quality improvement efforts on the chronic care model<sup>[40,41]</sup>. Indicators were selected from existing guidelines, a pilot study was conducted to collect data before and after the intervention in cases and controls, and results of the pilot study were used to refine the processes used for quality improvement. After the rigorous initial process, the network was expanded to additional centers. Initial research from this network demonstrated significant improvement in the completion of processes, which resulted in improved outcomes<sup>[41]</sup>.

IBD practitioners and researchers are certainly not

the only specialists dealing with these issues in chronic inflammatory conditions. No fewer than four sets of quality indicators have been developed for arthritis care<sup>[42-45]</sup>, including one set for juvenile idiopathic arthritis<sup>[43]</sup>. While quality improvement efforts are planned for arthritis care (including by the eumusc.net network), care providers also struggle with issues of measurement and validation<sup>[46]</sup>.

## CONCLUSION

The increased availability of routinely-collected health data (including disease registries, electronic health records, and health administrative data) has resulted in a spotlight on unnecessary variability in the medical care of children with IBD. Quality improvement efforts have therefore never been more relevant, and reduction in negative outcomes in children with chronic diseases such as IBD could save healthcare costs and improve long-term quality of life for patients and families. While quality improvement programs in pediatric IBD are more advanced than those in other pediatric diseases, continually learning from the successes and limitations of networks such as ICN will allow for more rapid improvement in outcomes. The development and validation of quality indicators that are more strongly associated with outcomes will allow for more efficient implementation of quality improvement efforts, thereby reducing costs while improving the quality of life of children with IBD. We are at the beginning of a revolution in health care improvement, and we must therefore continuously learn from and improve upon the methods currently employed by our current quality improvement efforts.

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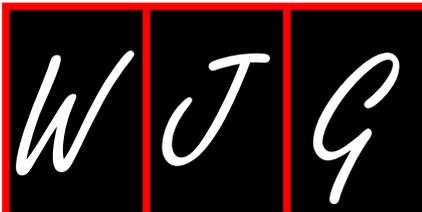
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## Interventions and targets aimed at improving quality in inflammatory bowel disease ambulatory care

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**Core tip:** Over the past decade, there has been increasing focus on improving quality in healthcare. This has led to the reinvigoration of the quality improvement movement. Inflammatory bowel disease is a complex, chronic condition with associated morbidity, health care costs, and reductions in quality of life. The condition is managed primarily in the outpatient setting. The delivery of high quality care is suboptimal in several ambulatory IBD domains. This review outlines current gaps in performance in IBD outpatient care and provides potential initiatives aimed at improvement.

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### Abstract

Over the past decade, there has been increasing focus on improving the quality of healthcare delivered to patients with chronic diseases, including inflammatory bowel disease. Inflammatory bowel disease is a complex, chronic condition with associated morbidity, health care costs, and reductions in quality of life. The condition is managed primarily in the outpatient setting. The delivery of high quality of care is suboptimal in several ambulatory inflammatory bowel disease domains including objective assessments of disease activity, the use of steroid-sparing agents, screening prior to anti-tumor necrosis factor therapy, and monitoring thiopurine therapy. This review outlines these gaps in performance and provides potential initiatives aimed at improvement including reimbursement programs, quality improvement frameworks, collaborative efforts in quality improvement, and the use of healthcare information technology.

### INTRODUCTION

Over the past decade, there has been increasing focus on improving the quality of healthcare. Much of this interest was inspired through the publication of *To Err is Human* by the Institute of Medicine (IOM) in 2000, that painted a portrait of a health care system full of preventable morbidity and mortality in desperate need for change<sup>[1]</sup>. This has led to the reinvigoration of the quality improvement (QI) movement, the foundation of which had developed over the last century.

QI is defined by the IOM as "the degree to which health services for individuals and populations increase

the likelihood of desired health outcomes and are consistent with current professional knowledge<sup>[2]</sup>. Fundamental principles of the study of QI include reflection on individual and peer performance in delivering high quality care, transparency in reporting performance, and implementing changes to improve deficiencies with the ability to measure successes and failures. Variation in practices may also be a marker of suboptimal performance. This had led to the resurrection and refinement of measures, study designs, and statistical analyses that are uniquely suited to QI.

Chronic disease management has become a significant focus of QI initiatives given their associated morbidity and cost. Some of this may be due to gaps in delivering evidence-based care. This was demonstrated in a landmark trial that showed that only 57% of outpatients regularly receive recommended standard of care for a variety of conditions<sup>[3]</sup>. As a result, there has been significant focus on improving delivery of evidence based care and preventative measures to patients with chronic disease in order to decrease complications, hospitalizations, and death. Moreover, quality indicators are increasingly becoming incorporated in the accreditation and funding models of healthcare institutions. Inflammatory bowel disease (IBD) is a chronic gastrointestinal condition characterized by relapsing inflammation. Crohn's disease (CD) and ulcerative colitis (UC) are the major subtypes of IBD. In North America, the incidence of CD ranges from 3.1-20.2 cases per 100000 population and 2.2-19.2 cases per 100000 population for UC<sup>[4,5]</sup>. While the incidence is less in Asia and the Middle East, the incidence and prevalence have been noted to be rising in many different regions of the world<sup>[5]</sup>. As in other chronic diseases, IBD patients are at increased risk of morbidity due to symptoms, hospitalizations, and complications of disease or therapy<sup>[6]</sup>. Moreover, there are significant health care costs and reduction in quality of life associated with IBD<sup>[7,8]</sup>. The economic burden of IBD is significant, with high disability rates among this young cohort of patients<sup>[9]</sup> and one cost analysis of eight European cohorts showing a mean total health care cost of 1871 euros per patient-year over 10 years<sup>[10]</sup>. Patients requiring hospitalization had 10 fold higher costs. Most patients with IBD are managed in the outpatient setting. However as disease severity progresses and complications of disease or therapy arise, hospitalization is often required. Unlike some other chronic conditions, IBD is a heterogeneous disease with a wide spectrum of disease phenotypes and management strategies. This makes disease wide QI strategies particularly challenging. Nonetheless, there are several areas of IBD care that are amenable to QI study and change. This review outlines current gaps in quality in a number of outpatient domains and provides potential initiatives aimed at improvement.

## ASSESSMENT OF DISEASE ACTIVITY

A challenging management issue in patients with IBD is how to best assess disease activity. This assessment has

traditionally been based on clinical symptoms. However, with the increasing number of more objective tools to assess disease activity now available, such as serum inflammatory markers and fecal calprotectin, the use of symptoms alone may no longer be the best approach to follow these patients. Reliance solely on symptoms can potentially miss ongoing inflammation that may not be clinically apparent. In a Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives (GETAID) study of 121 patients with CD, there was weak correlation between clinical symptoms and endoscopic activity<sup>[11]</sup>. This puts patients at risk of disease complications and may make treatment more difficult once symptoms ultimately develop. Alternatively, active inflammation may not always be the cause of persistent gastrointestinal symptoms in patients with IBD. A meta-analysis of 13 studies containing 1703 IBD patients found the pooled prevalence for symptoms meeting criteria for IBS was 39%, with an OR compared to healthy controls of 4.89 (95%CI: 3.43-6.98)<sup>[12]</sup>. Similarly, a pediatric study found significant overlap between functional abdominal pain and CD, with almost half of the patients meeting criteria for functional pain classified as having active IBD according to the Pediatric CD activity index<sup>[13]</sup>. This often leads to patients being inappropriately treated with immunosuppressants, with a low likelihood of improvement in symptoms and exposure to unnecessary risk. Therefore, there is a clear need for routine objective assessments of patients with IBD both at diagnosis and during follow up. While regular endoscopic evaluation, the gold standard to assess disease activity, has well established barriers such as cost and invasiveness, incorporating other objective tools such as erythrocyte sedimentation rate, C-reactive protein, and fecal calprotectin may facilitate more accurate and targeted approaches to managing these patients. A recent comparison of these tools noted a sensitivity and specificity of C-reactive protein > 6 mg/L of 68% and 72%, respectively as compared to a sensitivity and specificity of fecal calprotectin of 91% and 90%, respectively for the detection of endoscopically active disease<sup>[14]</sup>. More studies such as this are needed to provide more insight on the most valuable and cost-effective non-invasive approach to monitor disease activity in patients.

## STERIODS SPARING AGENTS

Corticosteroids are effective in inducing remission among patients with CD and UC<sup>[15]</sup>. However, they have not been shown to be helpful in long-term maintenance<sup>[16]</sup>. Moreover, their poor safety profile and tolerability makes avoidance of prolonged use a priority. Nonetheless, a significant proportion of patients treated with corticosteroids remain on extended courses. A retrospective review of patients referred to a tertiary IBD center in the United States found that over 75% of patients had been on corticosteroids for over 3 mo, including patients classified as having "mild" disease<sup>[17]</sup>. There was no attempt to consider steroid sparing medications, such as immunomod-

lators, in almost 60% of patients. Similarly, in a study of time trends in therapy among 16 medical centers between the years 1998 and 2005, there was a 27% increase in prolonged corticosteroid use (defined as > 120 d) among patients with UC<sup>[18]</sup>. Significant variation in the use of steroid-sparing agents was noted among centers. This was also demonstrated among 10 North American pediatric centers whereby the use of immunomodulators as a steroid sparing-agent varied significantly, ranging from 30%-95% of patients followed at the center<sup>[19]</sup>. Corticosteroids are a well-established risk factor for osteoporosis and as such, patients on extended courses should undergo bone density measurement. Despite this recommendation, a practice audit at a large tertiary IBD center found that almost 80% of patients referred had not received the appropriate screening for metabolic bone disease<sup>[17]</sup>. Clearly there is significant variation in practice patterns regarding the recognition of the need to minimize steroid exposure and highlights the underuse of steroid-sparing agent such as immunomodulators and anti-TNF therapy.

## SCREENING PRIOR TO ANTI-TUMOR NECROSIS FACTOR THERAPY

Anti-tumor necrosis factor therapy (anti-TNF) has emerged as an effective treatment for IBD<sup>[20-23]</sup>. It, however, carries risk of infection due to immunosuppression. The incidence of reactivation of latent tuberculosis infection (LTBI) has been shown to be increased among individuals treated with anti-TNF. A review of the United States Food and Drug Administration Adverse Event Reporting System found an incidence of 24 cases of tuberculosis per 100000 per year among those treated with anti-TNF, which translates into a 4 fold increased risk<sup>[24]</sup>. Similarly, the incidence of hepatitis B virus (HBV) reactivation is also increased among these patients<sup>[25-27]</sup>.

In order to minimize this risk, screening measures have been recommended prior to initiating anti-TNF therapy. Screening for LTBI and HBV prior to treatment has been recommended by the United States Food and Drug Administration, Health Canada, and all gastrointestinal societies<sup>[28-31]</sup>. Screening is effective in reducing infections complications, is easy to perform, and has minimal risks to patients<sup>[32-34]</sup>. This involves tuberculin skin testing and chest-X-ray for LTBI and a panel of three serological blood tests for HBV (HBsAg, HBsAb, HBcAb). Adherence to screening with tuberculin skin testing and chest x-ray has been shown to reduce the risk of tuberculosis by 78%-90%<sup>[32,33]</sup>. Screening for HBV with subsequent vaccination or chemoprophylaxis if indicated has also been shown to be effective<sup>[34]</sup>.

Despite these recommendations, cases of severe and sometimes fatal infection with tuberculosis or hepatitis B have been described, and many of these can be attributed to lack of screening<sup>[34-36]</sup>. A retrospective review of over 200 patients followed at a large United States academic IBD center revealed only 65% of patients were appropriately screened for tuberculosis and 25% screened for

hepatitis B<sup>[37]</sup>. Similarly, a study from Australia showed that only 50% of gastroenterologists were routinely screening for HBV prior to starting anti-TNF<sup>[38]</sup>. This underscores the problem in provider's adherence to screening. The development of tuberculosis or hepatitis B while on anti-TNF has the potential for high morbidity and mortality. Given the ease and effectiveness of screening and the consequences of lack of screening, one can argue that anti-TNF screening rates less than 100% are unacceptable.

There is growing literature exploring contributors to this safety problem. In their review of 287 IBD patients starting anti-TNF, Vaughn *et al.*<sup>[37]</sup> identified factors most often associated with lack of screening for tuberculosis: previous exposure to anti-TNF [OR = 5.3 (95%CI: 2.8-10.3)], health care providers in practice for more than 10 years [2.5 (95%CI: 1.4-4.5) and treatment at a non-IBD center [1.9 (95%CI: 1-3.4)]. The factors contributing to lack of HBV screening were the same. These reasons highlight the role of lack of knowledge, as physicians in practice longer or those at a non-IBD center may be less likely to be up to date with current guidelines. Previous exposure to anti-TNF may falsely reassure the prescribing physician that the appropriate work up had already been completed. This highlights the contribution of confusion as to who is responsible for screening. Uncertainty as to how and when to screen is also an important contributor, as evident in a gastroenterology practice audit that showed that while most knew that screening was indicated, there was significant heterogeneity in the type and timing of screening<sup>[38]</sup>. Thus, knowledge gaps as to the need for screening, confusion surrounding responsibility for screening, and details regarding how to screen appear to be major contributors to this problem.

## MONITORING THIOPURINE THERAPY

Thiopurines, including azathioprine and 6-mercaptopurine, are commonly used in patients with IBD. While most patients tolerate these medications with minimal side effects, ongoing monitoring is required once therapy commences. Regular complete blood counts (CBC) are recommended by all published guidelines to monitor for myelosuppression<sup>[39-42]</sup>, for example weekly CBC within the first month of therapy, every other week for the following two months, and every 3 mo thereafter. While the routine checking of thiopurine S-methyltransferase (TPMT) genotype and phenotype status prior to therapy remains controversial, it is strongly recommended by the United States Food and Drug Administration and has recently been listed as a quality indicator<sup>[31,43]</sup>. Regular monitoring of liver chemistries is also recommended by some, although the frequency of which is less clear<sup>[42]</sup>. Despite tremendous experience with this class of medication that has been available for over 5 decades, variation in monitoring patients while on this medication is significant and lapses in many best-practice recommendations are noted. A survey of members of the Canadian Association of Gastro-

**Table 1 American Gastroenterology Association Physician Quality Reporting System inflammatory bowel disease measures**

1	IBD type, location and activity all documented
2	Corticosteroid sparing therapy after 60 d
3	Bone loss assessment
4	Influenza immunization
5	Pneumococcal immunization
6	Testing for latent tuberculosis before initiating anti-TNF therapy
7	Assessment of Hepatitis B status before initiating anti-TNF therapy
8	Tobacco use: screening and cessation intervention

IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor.

enterology revealed that while all providers acknowledged the need to monitor blood counts, there were differences in the frequencies of monitoring<sup>[44]</sup>. Forty-two percent of those surveyed checked CBC weekly after starting therapy while 26% said they checked monthly and 23% biweekly during the initial period of treatment. Moreover, only 62% of respondents routinely monitored liver chemistries. In terms of routine TPMT testing, an international questionnaire sent to experts in the use of thiopurines in IBD found that only 30% and 43% routinely ordered genotype and phenotype testing, respectively<sup>[45]</sup>. Lack of reimbursement for testing was the most important predictor of not ordering the test, and almost half of respondents felt that they would incorporate routine testing into their practice if it was reimbursed.

More recently, an association with thiopurine use and non-melanoma skin cancer (NMSC) has been noted. In a review of over 1000 South African IBD patients, a strong association was noted between thiopurine exposure and NMSC (OR = 5.0, 95%CI 1.1-22.8)<sup>[46]</sup>. This was similar to the association noted by Peyrin-Biroulet *et al*<sup>[47]</sup> in which ongoing thiopurine use had a hazard ratio for NMSC development of 5.9 (95%CI: 2.1-16.4). Lifelong, regular dermatologic screening has therefore been recommended<sup>[48]</sup>. Nonetheless, a recent survey of dermatologists and gastroenterologist found that only 46% of gastroenterologists were aware of the association between NMSC and immunosuppression<sup>[49]</sup>. This implies that at least half of IBD patients are not receiving the recommended screening.

## INTERVENTIONS AIMED AT IMPROVEMENT

In order to adequately address gaps in care, an understanding of the contributing factors to the target problem is essential. While the evidence in support of a potential intervention is often regarded as the most important factor when choosing between potential initiatives focused on improving care, there is often limited supporting research available. As a result, other factors also need to be considered when choosing QI interventions including the prevalence and severity of the problem, the potential for undesirable outcomes as a result of the intervention, cost, complexity, and the ability to generate momentum

for future related initiatives<sup>[50]</sup>. Moreover, once an intervention has been selected, continuous measurement is essential in order to know if an observed change represents an improvement. Thus, prior to implementing an initiative, well defined measures need to be developed and measured continuously. This will provide support that the initiative is responsible for any observed improvements in performance or alternatively, negative outcomes and unattended consequences.

### Reimbursement programs

Guidelines have outlined algorithmic approaches for following this complex group of patients. However, the uptake of IBD guidelines by gastroenterologists has been shown to variable<sup>[51,52]</sup>. Therefore, other improvement approaches are necessary. In 2006, the American Gastroenterology Association began to develop quality indicators that would be eligible for reimbursement through the Physician Quality Reporting System (PQRS)<sup>[53]</sup>. Recently, IBD specific measures have been added to this growing list of indicators, and documentation of disease activity was the first such IBD indicator implemented. Other IBD indicators eligible for reimbursement through this program include recommending steroid-sparing therapy after 60 d of corticosteroid, assessment of tuberculosis and hepatitis B status prior to anti-TNF therapy, vaccinations, bone loss assessment, and addressing tobacco cessation (Table 1). While the impact of the PQRS on increasing objective assessment of disease activity is not yet known, data extrapolated from other disease states shows promise for the potential beneficial impact of similar reimbursement programs<sup>[54]</sup>. Nonetheless, prior to implementing such an intervention, careful study is required as the literature showing the benefits of reimbursement programs on quality are conflicting and some studies identifying unintended consequences, such as providers avoiding the most severely ill patients, a phenomenon known as “adverse selection”<sup>[54-57]</sup>.

Although not designed for the purposes of a reimbursement program, the Crohn’s and Colitis Foundation of American have recently sponsored the publication of a set of quality indicators<sup>[43]</sup>. Both process and outcome indicators were developed that encompass a variety of domains in IBD care including treatment, surveillance, and health care maintenance. A number of corticosteroid related indicators are defined such as “IF a patient with IBD requires at least 10 mg prednisone (or equivalent) for 16 wk or longer, THEN an appropriately dosed steroid-sparing agent or operation should be recommended” and steroid related outcomes measures including; (1) proportion of patients with steroid-free clinical remission for a 12 mo period; and (2) the proportion of patients currently taking prednisone. Screening for latent tuberculosis and hepatitis B prior to therapy with anti-TNFs and TPMT testing prior to thiopurine therapy are also included. As more quality indicators develop and become increasingly incorporated into the accreditation processes of health care institutions, it is likely that more reimbursement models, or alternatively citations and pen-

alties for under performance, can be expected over the coming years.

### **Quality improvement frameworks**

It does not appear that knowledge gaps are solely responsible for barriers in delivering high quality, evidence-based care. In terms of the underuse of steroid sparing agents, for example, the avoidance of prolonged corticosteroid and the importance of transitioning to steroid-sparing agents are not new concepts, have been endorsed by all gastrointestinal societies, and have been highlighted in guidelines for many years. This was borne out in a survey of gastroenterologists from 36 countries whereby 100% of those surveyed agreed that there is minimal evidence for continuing high dose corticosteroids for more than 3 wk and that steroid-sparing agents should begin to be considered after 2-4 wk of therapy<sup>[58]</sup>. Therefore other contributors beyond physician knowledge base need to be addressed. Patients often initiate or modify steroid doses on their own without consultation with their health care provider. This may be due to poor access to a timely visit to a gastroenterologist when symptoms first present or when disease activity flares. Early referral to a specialist has been shown to improve IBD outcomes and initiatives aimed at improving access to gastroenterology have been shown to reduce steroid use and increase the use of early steroid-sparing therapy<sup>[59]</sup>. A Swedish gastroenterology unit implemented a quality improvement framework whereby a registry of quality metrics was established and performance tracked<sup>[60]</sup>. All routine visits were initiated by the clinic, rather than the patients and regular reminders to contact a designated IBD nurse for problems was provided. The program resulted in 98% of patients receiving regular IBD follow up visits, less than 3 wk between primary care referral and specialist visit, and less than 2 d to schedule an acute patient visit during disease flares. This experience highlights that implementing well designed frameworks, which are common place in other chronic diseases, has the potential to improve quality of care in IBD<sup>[61]</sup>. Frameworks need to be developed with the appropriate local context in mind with and some have argued that frameworks do nothing to improve quality but rather improve documentation alone<sup>[62]</sup>. This underscores the importance of continuous measurement after implantation to ensure the effort and costs associated are translating to improvements.

### **Collaborative efforts in quality improvement**

Another potential motivator for change is collaborative efforts between institutions. These involve multiple sites working together towards a common improvement goal through receiving training in quality improvement methods, defining QI metrics, tracking performance, and transparency in reporting<sup>[63]</sup>. While the use of improvement collaboratives in inflammatory bowel disease lags behind other chronic diseases, early outcomes of such initiatives have been promising. The ImproveCareNow Network consists of 51 pediatric hospitals across the United States and Europe that adopted the Chronic Ill-

ness Care Model and developed standardized practices and measures<sup>[64,65]</sup>. Process and outcomes measures were prospectively collected and shared between sites. Early data has shown significant improvements in processes of care and patient outcomes in a variety of care areas. The use of a classification bundles to assess disease location, phenotype, activity, and nutritional/growth parameters at every visits has allowed for standardization between sites. Not only does this improve care, but also allows for collaborative clinical research efforts. Other outcomes already reported by the network include a decrease in the number of patients with CD on corticosteroids and an increase in the number of patients starting thiopurines with TPMT activity measured. These improvements in process measures are likely responsible for the increased remission rates noted in the participating sites. While more data on the efficacy of this and other such collaboratives are needed, given that an overarching theme of QI is to improve care delivery throughout the entire health care system, more widespread adoption of such broad, multi-site quality improvement initiatives should be considered.

### **Advancing healthcare information technology**

The widespread incorporation for healthcare information technology (HIT) has been identified as essential in order to improve quality, safety, efficiency, and coordination of care by many leaders in the field of QI and patient safety<sup>[66]</sup>. Many of the organizations regarded as leaders in the field of QI, such as the Veterans Affairs (VA) system in the United States or the Intermountain Healthcare Network in Utah attribute their success to the early adoption of electronic medical records and ongoing refinement of HIT resources<sup>[67]</sup>. Providers delivering care to IBD patients have the potential to benefit from a variety of HIT related interventions including an electronic health record, computerized provider order entry (CPOE), and clinical decision support. If designed well and appropriately adapted to the context of a given institution, an electronic health record has the potential to improve efficiency, safety, and communication. It also has the potential to engage patients as platforms in which patients are able to access their own health record are increasingly being developed<sup>[66]</sup>. This is important in IBD as patients are often young and may travel or move frequently for school and work. An electronic record also lends well to automated reminders which could address many areas of care that have been shown to have suboptimal performance such as monitoring blood work on thiopurines and bone density assessments<sup>[63]</sup>. CPOE is another HIT intervention that in addition to decreasing medication errors, has the potential to enable drug interaction warnings, monitoring tests, and linkage to decision support systems<sup>[66]</sup>. For example, order sets involve a collection of orders or investigations at one location that when designed well, are effective through improving efficiency, decreasing variation, enhancing workflow, and improving communication of evidence based practices<sup>[68,69]</sup>. Traditionally, order sets have been paper-based, but

electronic order sets have become increasingly popular and have already been evaluated extensively in the patient safety literature. Compared to traditional paper order sets, electronic order sets have been shown to be more readily accessible, easier to link with other relevant order sets, and can be updated in real time<sup>[70]</sup>. A number of areas within IBD patient care may be improved with electronic order sets, such as pre anti-TNF screening. While the evidence for order sets improving anti-TNF screening is lacking, examples in other fields support their utility. A pediatric study showed that an order set improved adherence to evidenced based asthma medication behaviors by almost 25%<sup>[71]</sup>. While these results are encouraging, the quality of most studies evaluating order sets is not high and often employ simple before and after designs with poor control of biases<sup>[72]</sup>. Moreover, some studies have shown unintended consequences of HIT. For example, one study aimed at using an electronic reminder to improve adherence to colon cancer found that following the intervention was unveiled, colon cancer screening adherence actually declined as a result of ineffective reminders and increased fecal occult blood screening rather than colonoscopy<sup>[73]</sup>. Nonetheless, the theory behind order set effectiveness is sound and addressed several of the contributors to the anti-TNF safety problem identified above including knowledge gaps and confusion with details as to how to screen.

## CONCLUSION

Caring for patients with IBD can be challenging due to the heterogeneous nature of the disease and the lack of consensus in many areas of practice. Variation in practice is therefore unavoidable and does not necessarily imply deficiencies in quality. Nonetheless, there are several aspects of IBD care whereby suboptimal performance has been documented and may be amenable to quality improvement initiatives including regular objective assessments of disease activity, recommending steroid sparing therapy, and appropriate monitoring of patients initiating and ongoing immunomodulator and anti-TNF therapy. Reimbursement programs, chronic disease frameworks, QI collaboratives, and health information technology resources are several potential interventions that may benefit IBD patient care. Quality performance indicators are expected to increasingly become incorporated into accreditation and funding models and it is therefore important that gastroenterologists become familiar with QI concepts and consider implementing initiatives where warranted.

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## Personalizing therapies for gastric cancer: Molecular mechanisms and novel targeted therapies

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### Abstract

Globally, gastric cancer is the 4<sup>th</sup> most frequently diagnosed cancer and the 2<sup>nd</sup> leading cause of death from cancer, with an estimated 990000 new cases and 738000 deaths registered in 2008. In the advanced setting, standard chemotherapies protocols acquired an important role since last decades in prolong survival. Moreover, recent advances in molecular therapies provided a new interesting weapon to treat advanced gastric cancer through anti-human epidermal growth factor receptor 2 (HER2) therapies. Trastuzumab, an anti-

HER2 monoclonal antibody, was the first target drug in the metastatic setting that showed benefit in overall survival when in association with platinum-5-fluorouracil based chemotherapy. Further, HER2 overexpression analysis acquired a main role in predict response for trastuzumab in this field. Thus, we conducted a review that will discuss the main points concerning trastuzumab and HER2 in gastric cancer, providing a comprehensive overview of molecular mechanisms and novel trials involved.

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**Key words:** Gastric cancer; Human epidermal growth factor receptor 2; Biomarkers; Target therapies; Trastuzumab; Lapatinib; Pertuzumab; Trastuzumab-DM1; Afatinib

**Core tip:** Advanced gastric cancer is a very aggressive disease though the standard chemotherapy protocols available. In 2010, Trastuzumab for Gastric Cancer trial showed that the combination of trastuzumab, could be considered a new standard option for patients with human epidermal growth factor receptor 2 (HER2) positive advanced gastric and gastro-esophageal junction cancer. Thus, a new era emerged for those patients due to the interesting possibility in prolong survival in a personalized setting (HER2 positive). Our manuscript will provide an overview of the molecular mechanisms involved and also promising targeted therapies in this field.

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## INTRODUCTION

Gastric cancer has been described since 3000 BC in ancient Egypt. One of the first epidemiologic studies on cancer, with data spanning from 1760 to 1839, pointed gastric cancer as the most common and lethal. In modern times, it remains one of the most important forms of cancer, with different geographic, ethnic and socio-economic distributions. Incidence is particularly high in Japan, China, South Korea, Chile and Costa Rica. The large regional variations in incidence possibly reflect different prevalences of *Helicobacter pylori* infection, which is responsible for more than 60% of gastric cancer globally. Globally, gastric cancer is the 4<sup>th</sup> most frequently diagnosed cancer and the 2<sup>nd</sup> leading cause of death from cancer, with an estimated 990000 new cases and 738000 deaths registered in 2008<sup>[1]</sup>. The human epidermal growth factor receptor 2 (HER2) protein, a 185 kDa protein (p<sup>185</sup>) encoded by a gene located on chromosome 17q21 is a transmembrane tyrosine kinase receptor with an extracellular ligand-binding domain; a short transmembrane domain and an intracellular domain with kinase activity (Figure 1). It belongs to the epidermal growth factor receptor (EGFR) family of growth factors comprising four structurally related members, HER1 or ErbB1, also known as EGFR, HER2 or ErbB2, HER3 or ErbB3 and HER4 or ErbB4. Activation occurs through homo- or heterodimerization induced by ligands. HER2 is designated an orphan receptor which is believed to homodimerize independently of a ligand or to heterodimerize with another ligand-bound member of the EGFR family. Activation triggers a cascade of events that involves autophosphorylation and activation of the tyrosine kinase domain, Ras/Raf/mitogen-activated protein kinase pathway, phospholipase C- $\gamma$  and phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) (Figure 2). HER2 receptors have also been found in nuclear localization, where they act as transcription factors for cyclin D1 and p53<sup>[2,3]</sup>. Therefore, *HER2* (also known as *c-erbB-2/neu*) acts as an oncogene involved in the regulation of cell proliferation, differentiation, motility and apoptosis<sup>[4-8]</sup>. Heterodimers of HER2 with other members of the HER family, particularly with HER3, are the most mitogenic dimers and HER2 increases the affinity of EGFR, HER3 and HER4 to their ligands<sup>[9-12]</sup>.

Analysing the molecular structure of HER2 allows new insights into approaching it as a potential therapeutic target (Figures 1 and 3). The extracellular domain of the receptor is subdivided into four subdomains. Whereas subdomains II and IV are involved in the process of dimerization, subdomains I and III are the binding sites for pertuzumab and trastuzumab respectively, two of the most well studied HER2 inhibitors which will be discussed further on. The transmembrane domain of HER2 plays an important role in the process of dimerization and oncogenic mutations in this region were described. The intracellular domain contains the active enzyme site which activates different downstream pathways by phosphorylation<sup>[13-16]</sup>.

The importance of addressing HER2 as a therapeutic target is underscored by a number of molecular and pathological findings. Amplified HER2 relates to processes of carcinogenesis and adverse pathologic features such as tumor size, invasion and metastatic spread; the level of *HER2* gene expression is much higher in cancer cells than that in non-malignant adult cells<sup>[17]</sup>. HER2 overexpression has been reported in breast, lung, salivary gland, ovary, colon, prostate and pancreatic cancers<sup>[18,19]</sup>.

About 10%-34% of invasive breast cancers present HER2 overexpression. Trastuzumab has shown survival advantage in early and metastatic disease and is now a part of standard care. HER2 overexpression stands as a poor prognosis marker for chemo- and endocrine therapy but at the same time as a positive predictive marker for treatment with trastuzumab. Furthermore, trastuzumab proved to be effective as adjuvant treatment in breast cancer with HER2 overexpression, with different chemotherapy regimens<sup>[20-26]</sup>. In gastric cancer, the prognostic role of HER2 overexpression remains controversial. The most important prognostic factor for gastric cancer is the tumor node metastasis (TNM) stage<sup>[20,27]</sup>. Initial works addressing the prognostic significance of HER2 overexpression reported a negative effect on overall survival (OS)<sup>[28,29]</sup>. However, conflicting results regarding the prognostic value of HER2 have been published more recently. Some studies found a negative effect of HER2 on prognosis with reduction in OS<sup>[17,20,29-36]</sup>, others found no relationship<sup>[37-40]</sup> and a trend towards improved survival was found in one cohort<sup>[41]</sup>. A comprehensive review by Jørgensen *et al.*<sup>[42]</sup> found that the majority of publications that fulfilled the selection criteria for the analysis, associated HER2-positive status with poor survival and clinicopathological characteristics such as serosal invasion, lymph node metastases, disease stage or distant metastases. Chua *et al.*<sup>[43]</sup> recently reviewed 49 studies with data regarding the relation of HER2 with clinicopathological variables and survival and concluded that HER2 overexpression is associated with poor survival; results pertaining other variables were not conclusive. HER2 overexpression has also been suggested as a molecular abnormality in the development of intestinal type gastric cancer and HER2 expression increases with disease progression, leading to the suggestion that the initial timing of this event probably occurs in early stages. Barros-Silva *et al.*<sup>[20]</sup> found overexpression and amplification in both components of mixed tumors (intestinal and diffuse components) and *HER2* amplification in early stages, supporting this idea of amplification in an early stage of carcinogenesis. Further support arises from the high levels of concordance between primary tumors and paired metastatic sites found by some authors, suggesting HER2 amplification as an early event and not acquired at a later moment by cells with metastatic potential<sup>[44]</sup>. Kataoka *et al.*<sup>[45]</sup> found no HER2 positivity in the diffuse component of mixed type cases, but also found HER2 overexpression in early TNM T1a cases, pointing towards an early event<sup>[30,46]</sup>. Although these data tend to establish

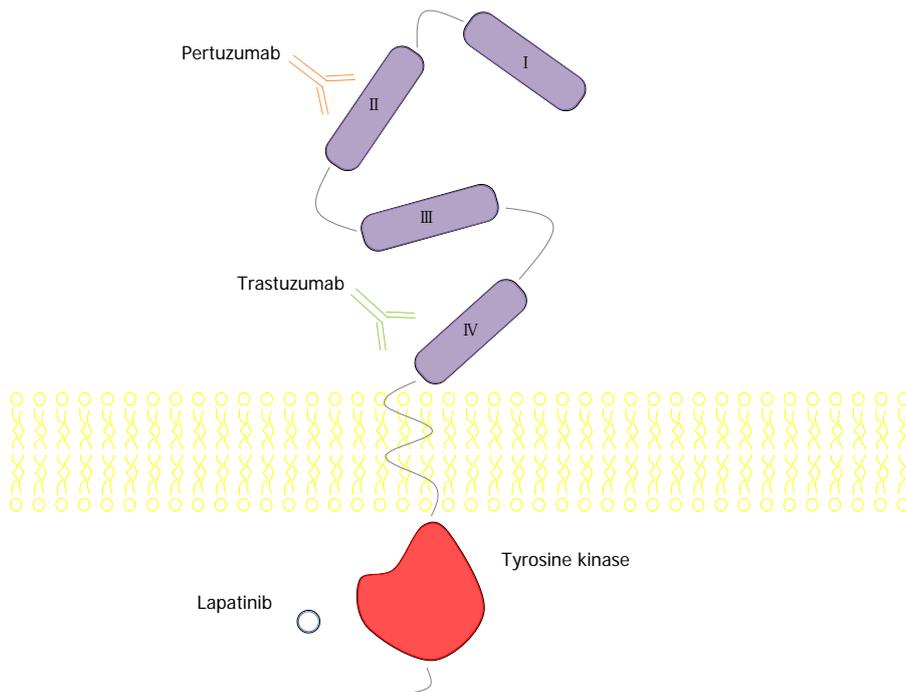


Figure 1 Human epidermal growth factor receptor 2 and binding sites. I-IV: Epidermal growth factor receptor 1-4.

HER2 as a potential negative prognostic factor in gastric cancer, the relation seems not to be as consistent as in breast cancer<sup>[42]</sup>. In fact, more recent studies demonstrate no significant prognostic relationship. In a study involving 381 metastatic gastric cancer patients, Yanjigian *et al*<sup>[47]</sup> found that patients with HER2-positive gastric cancer had longer median OS compared with HER2-negative gastric cancer patients, but on multivariate analysis HER2 status was not an independent prognostic factor. Terashima *et al*<sup>[48]</sup> found no correlation with OS in 829 stage II/III resected gastric cancer patients. Hsu *et al*<sup>[49]</sup> investigated 1036 gastric cancer patients undergoing curative-intent resection. Although HER2 positivity emerged as a favourable prognostic factor for stage III-IV gastric cancer on univariate analysis, it did not on multivariate adjustment.

Despite these conflicting results, it seems likely that HER2 is not associated with an adverse prognosis in gastric cancer in an extent similar to breast cancer; nevertheless, inhibition of the HER2 pathway in patients with HER2 amplification demonstrated clinical benefits. In this review, we will address the main advances in the treatment of advanced gastric cancer, focusing on the novel biomarkers and target therapies concerning HER2 signalling pathways.

## HER2 TESTING IN GASTRIC CANCER

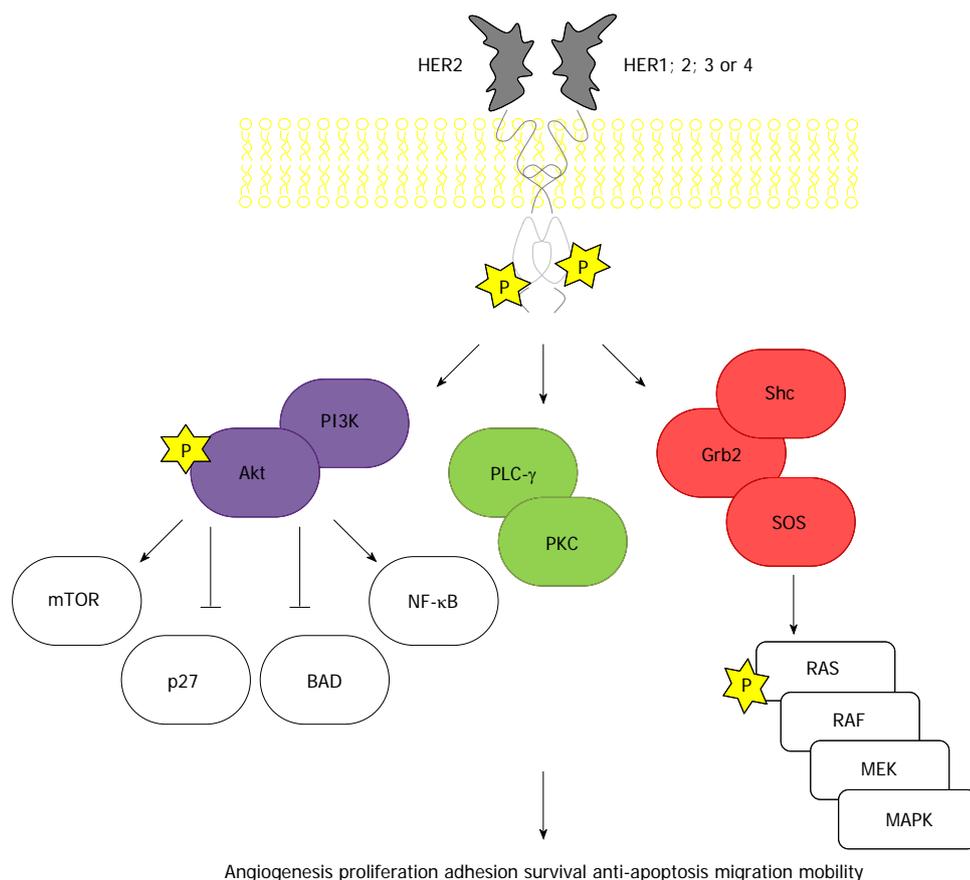
A precise analysis is necessary in order to address the status of HER2 expression in gastric cancer, which constitutes an essential step to select the patients feasible to treatment with HER2 target therapies. Techniques include primarily immunohistochemistry (ICH) and *in situ* hybridization (ISH), which constitute standard techniques

also used in the current practice of HER2-status determination in breast cancer. Current evidence suggests the need to adopt the methods used in breast cancer in order to address HER2 expression in gastric cancer<sup>[50]</sup>. Considering the different biologic origin of the tissue, the high density of glandular structures needs to be understood in its context. In gastric tissue, ICH staining for HER2 occurs typically on the basolateral membrane and less so on the luminal aspect of the cell, conferring an U-shaped appearance to the staining whereas completeness of the membrane staining is the rule for higher scores in breast cancer. Another difference concerns the heterogeneity of immunostaining which is rare in breast, but frequent in gastric tumors. ICH should be used as primary test; cases with ICH score 3+ are candidates for HER2 directed therapy, 2+ scoring cases should be re-tested using ISH; in the case of ISH positivity patients are eligible for these therapeutic modality<sup>[51]</sup>.

## HER2-DIRECTED THERAPIES IN GASTRIC CANCER

In January 2010, the European Medicines Agency granted approval to trastuzumab plus chemotherapy in the treatment of with IHC 3+ or 2+/metastatic adenocarcinoma of the stomach or gastro-esophageal junction (GEJ)<sup>[52]</sup>. The United States Food and Drug Administration approved trastuzumab for HER2 overexpressing patients, without further specification<sup>[53]</sup>.

Trastuzumab is a fully humanized monoclonal antibody that binds to the extracellular domain of the receptor, acting by blockage of the HER2 receptor cleavage, inhibition of dimerization, as well as by the induction



**Figure 2 Human epidermal growth factor receptor 2 signalling pathways.** HER: Human epidermal growth factor receptor; PI3K: Phosphoinositide 3-kinase; BAD: Bcl-2-associated death promoter protein; NF-κB: Nuclear factor κB; PLC-γ: Phospholipase C gamma 1; PKC: Protein kinase C; Grb2: Growth factor receptor-bound protein 2; SOS: Son of Sevenless; MEK: Mitogen-activated protein kinase 1; MAPK: Mitogen-activated protein kinase; mTOR: Mammalian target of rapamycin; RAS: Rat sarcoma viral oncogene; RAF: Rapidly accelerated fibrosarcoma.

of antibody-dependent cellular cytotoxicity (ADCC), increasing endocytosis of the receptor and possibly through anti-angiogenic effects<sup>[54-56]</sup>. It was developed in the 1990s, after murine monoclonal antibodies directed to the extracellular domain of HER2 were produced and evaluated in cell lines and xenografts<sup>[57-59]</sup>.

**Preclinical data**

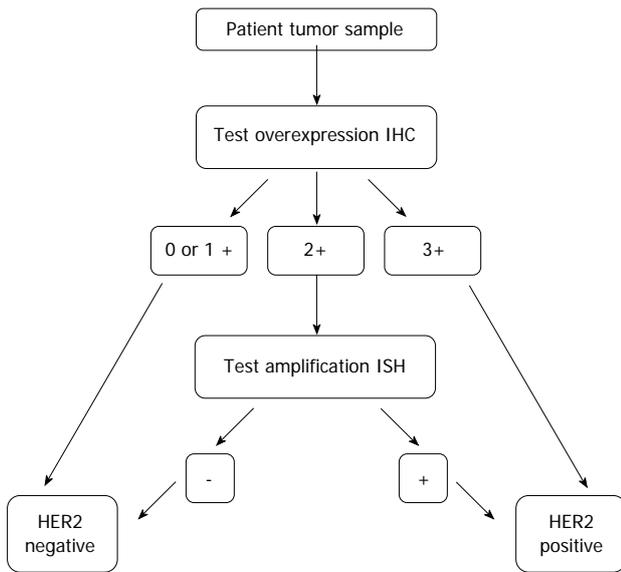
Overexpression of HER2 in gastric cancer cells was first reported in 1986 by Sakai *et al*<sup>[60]</sup> and Fukushima *et al*<sup>[61]</sup>. Preclinical models of gastric cancer were successful in demonstrating the inhibitory effect of trastuzumab on human gastric cancer cell lines *in vitro* and in mice xenografts *in vivo*, with additive and synergistic antineoplastic effects in combination with chemotherapy<sup>[59,62-65]</sup>. A study by Tanner *et al*<sup>[17]</sup> points out a gastric cancer cell line that was as sensitive to trastuzumab as a breast cancer cell line, both of them with amplified *HER2*, while Matsui *et al*<sup>[63]</sup> reported suppression of tumor growth in a xenograft model. Enhanced antineoplastic effects were observed with capecitabine, cisplatin, docetaxel, paclitaxel and irinotecan<sup>[62]</sup>, and a further synergistic effect with cisplatin has been found by Kim *et al*<sup>[64]</sup>.

**Clinical data**

Although information on the specific pathways involved

is scarce, *HER2* has been shown to be amplified in gastric cancer and *HER2* is progressively regarded as an important biomarker and driver of cancerization in gastric cancer, with studies pointing out amplification or overexpression in 7%-34% of tumors, mainly in the intestinal type and in GEJ and proximal tumors<sup>[17,27,66]</sup>.

Cortés-Funes *et al*<sup>[67]</sup> presented preliminary results of a phase II study involving 21 chemotherapy-naïve patients with *HER2* overexpressing locally advanced or metastatic gastric cancer. Trastuzumab at a loading dose of 8 mg/kg and maintenance dose of 6 mg/kg and cisplatin 75 mg/m<sup>2</sup> were administered every 21 d until progression, unacceptable toxicity or withdrawal of consent. Response rate was of 35%, with 17% of patients achieving stabilization. The tolerability profile was favourable; no grade 4 toxicity was observed and most the frequent grade 3 events were asthenia, nausea or vomiting, diarrhea, hyporexia and neutropenia. Data from another preliminary phase II study involving 16 gastric cancer patients were presented by Egamberdiev *et al*<sup>[68]</sup>. Trastuzumab 6 mg/kg was administered once in addition to cisplatin 100 mg/m<sup>2</sup> during 3 d + fluorouracil (5-FU) 1000 mg/m<sup>2</sup> 3 d + leicovirin 100 mg/m<sup>2</sup> 3 d, every 3 wk. Authors reported an objective response rate of 54.5% in the combined therapy group *vs* 33.3% in the chemotherapy-only group and a median remission duration of 8.3 mo *vs* 5.2 mo. In a recent phase



**Figure 3** Human epidermal growth factor receptor 2 testing algorithm. IHC: Immunohistochemistry; HER2: Human epidermal growth factor receptor 2; ISH: *In situ* hybridization.

II study carried out by Grávalos *et al.*<sup>[69]</sup>, chemo-naïve patients with non-resectable advanced or metastatic gastric or GEJ adenocarcinoma overexpressing HER2 were treated with trastuzumab 8 mg/kg as loading dose and 6 mg/kg in subsequent cycles + cisplatin 75 mg/m<sup>2</sup> every 3 wk. Twenty-two out of 228 patients (9.6%) enrolled had HER2 overexpression. An overall response rate of 32% was found, with disease control achieved in 64% of patients; median time to progression was 5.1 mo. No grade 4 toxicities occurred, whereas most frequent grade 3 adverse events were asthenia, neutropenia, anorexia, diarrhoea and abdominal pain. Interestingly, higher baseline HER2 extracellular domain levels associated with better response to therapy.

In more recent studies, HER2 overexpression was found to be lower than previously reported, especially in distant gastric cancers<sup>[70]</sup>. Resectable gastric cancer has reported HER2-positive ratios of 8.1% and 11.7%, suggesting that in resectable gastric cancer HER2 positive status might be less frequent than in advanced gastric cancer<sup>[71,72]</sup>.

The phase III ToGA trial constitutes a milestone, establishing trastuzumab as the first biological therapy that demonstrated survival benefits in gastric cancer<sup>[62,63]</sup>. ToGA was a multicenter, international trial, undertaken in 24 countries<sup>[73]</sup>. It evaluated the combination of trastuzumab with standard chemotherapy (cisplatin + either capecitabine or 5-FU) in advanced (inoperable locally advanced, recurrent or metastatic) HER2-positive gastric cancer as a first-line therapy *vs* chemotherapy alone. Patients were treated with six cycles of chemotherapy in both treatment arms, with patients in the experimental arm continuing to be treated with trastuzumab until disease progression. Cisplatin 80 mg/m<sup>2</sup> was given on day 1 by intravenous infusion. Capecitabine 1000 mg/m<sup>2</sup> was given orally twice a day for 2 wk followed by a 1-wk rest

or 5-FU 800 mg/m<sup>2</sup> per day was given by continuous infusion on days 1-5 of each cycle. Trastuzumab was given intravenously at a loading dose of 8 mg/kg on day 1 of the first cycle, followed by 6 mg/kg afterwards.

The primary objective of the study was to compare OS in both arms, and the secondary objectives were to compare progression-free survival (PFS), time to progression, overall response rate, disease control, duration of response and quality of life between the two treatment arms. Among 3665 tumor tissue specimens screened for HER2 positivity, 22% were HER2 positive (34% of the intestinal type *vs* 6% of diffuse and 20% of mixed types). Assessment was done with IHC and fluorescence ISH (FISH), according to Figure 3. The highest rate was observed in 34% of GEJ cancer and 20% of gastric cancer samples<sup>[74]</sup>, which is in conformity with other studies where positivity rates for the GEJ are between 24%-35% and in gastric cancer samples comprise 9.5%-21.0%<sup>[17,27,75-77]</sup>.

The combination of trastuzumab with chemotherapy in advanced HER2-positive cancer patients led to significantly better OS compared to the same chemo-therapeutic regimen alone (median OS in the combination therapy group was 13.8 mo against 11.1 mo in the chemotherapy-alone group). This effect was observed in patients with intestinal type gastric cancer but not in those with diffuse type gastric cancer<sup>[73,78]</sup>. Median PFS (6.7 mo *vs* 5.5 mo) and radiological response rate (47% *vs* 35%), also improved with trastuzumab therapy. Exploring these data further, a sub-group analysis of the ToGA study which excluded patients with IHC 0-1+ FISH+ disease, found a main gain in median survival of 4.2 mo, comparable to the figures in breast cancer<sup>[28]</sup>. In fact, patients with strongest HER2 expression (IHC 3+ FISH+) gained the greatest benefit, with a median survival of 17.9 mo in patients treated with trastuzumab *vs* 12.3 mo with chemotherapy alone.

A summary of selected clinical trials of trastuzumab in gastro-esophageal cancer can be found in Table 1.

### Adjuvant treatment

In this behalf, it is important to consider the possible benefits of trastuzumab in the adjuvant setting for earlier stages of the disease; however activity of targeted therapeutics in advanced disease should not automatically be extrapolated into the adjuvant setting, as results may be misleading<sup>[79]</sup>. Trials have been initiated which intend to investigate anti-HER2 therapeutics in this setting<sup>[80,81]</sup>. Early onset gastric cancer (presenting at or under the age of 45) seems to have lower HER2 overexpression than in late onset cases, with possible different molecular genetic pathways<sup>[82-84]</sup>. Ongoing clinical trials with trastuzumab can be found in Table 2.

### Maintenance therapy

From a clinical perspective, data known from breast cancer suggest that trastuzumab administration after disease progression might have benefits in OS<sup>[23,85]</sup>. In an observational study of 623 patients, median time to progression was longer in patients who continued trastu-

**Table 1** Selected clinical trials of trastuzumab in gastro-esophageal cancer

Ref.	Phase	Treatment	n	OS (mo)	PFS (mo)	RR	CR	PR
Bang <i>et al</i> <sup>[73]</sup>	III	5-FU + cisplatin or capecitabine + cisplatin	290	11.1	5.5	34.50%	NA	NA
		Trastuzumab + 5-FU + cisplatin or trastuzumab + capecitabine + cisplatin	294	13.8	6.7	47.30%	NA	NA
Cortés-Funes <i>et al</i> <sup>[67]</sup>	II	Trastuzumab + cisplatin	21	NA	NA	41.10%	5.80%	35.00%
Egamberdiev <i>et al</i> <sup>[68]</sup>	II	Trastuzumab + leucovorin + cisplatin + 5-FU	16	NA	8.3	54.50%	NA	NA
		Leucovorin + cisplatin + 5-FU	18	NA	5.2	33.30%	NA	NA
Grávalos <i>et al</i> <sup>[69]</sup>	II	Trastuzumab + cisplatin	22	NA	5.1	32.00%	NA	NA

OS: Overall survival; PFS: Progression-free survival; 5-FU: 5-fluorouracil; RR: Response rate; CR: Complete response; PR: Partial response; NA: Not available.

**Table 2** Clinical trials with trastuzumab-based combination therapies

Setting, therapy line	ID	Phase	n	Treatment combined with trastuzumab	Primary EP	Status
Operable disease	NCT01196390	III	480	Carboplatin, paclitaxel, radical radiotherapy	PFS	Recruiting
	NCT01472029	II	53	5-FU, leucovorin, docetaxel, oxaliplatin	Rate of CR	Recruiting
	NCT01130337	II	45	Oxaliplatin, capecitabine	PFS	Active, not recruiting
Advanced first line	NCT01450696	III	400	Cisplatin, capecitabine	OS	Recruiting
	NCT01503983	II	51	Oxaliplatin, capecitabine	OS	Recruiting
	NCT01461057	II	30	Cisplatin, capecitabine, pertuzumab	Safety	Active, not recruiting
	NCT01396707	II	56	Oxaliplatin, capecitabine	RR	Recruiting
	NCT01364493	II	51	Oxaliplatin, capecitabine	RR	Recruiting
	NCT01359397	II	80	Docetaxel, oxaliplatin, capecitabine, bevacizumab	PFS	Recruiting
	NCT01228045	II	30	Cisplatin, S-1	RR	Unknown
	NCT01191697	II	36	Oxaliplatin, capecitabine, bevacizumab	RR	Recruiting
Advanced second line	NCT01402401	II	48	AUY922	RR	Terminated

ID, NCT identification (information available at <http://clinicaltrials.gov>, as accessed June 28, 2013). OS: Overall survival; PFS: Progression-free survival; 5-FU: 5-fluorouracil; RR: Response rate; CR: Complete response; PR: Partial response; NA: Not available.

zumab beyond progression than in those who stopped (10.2 mo *vs* 7.1 mo)<sup>[86]</sup>. Data from an interventional study involving 156 patients revealed OS rates of 20.4 mo *vs* 25.5 mo and response rates of 27.0% *vs* 48.1% in patients who stopped and continued trastuzumab beyond progression, respectively. Continuation of trastuzumab beyond progression was not associated with increased toxicity<sup>[87]</sup>. However, the issue is still a matter of debate, as increasing therapeutic options pose a challenge on the best possible sequencing and combinations of these interventions<sup>[88-90]</sup>.

### Perioperative treatment

Perioperative chemotherapy regimens have shown promising results in gastric cancer. The MAGIC trial randomized over 500 patients to either surgery alone or perioperative chemotherapy consisting of epirubicin, cisplatin and fluorouracil (3 cycles before and 3 cycles after surgery). This triplet therapy demonstrated a decrease in tumor size and improved PFS and OS in comparison with surgery alone<sup>[91,92]</sup>. In addition, some data indicate that response to neoadjuvant treatment is a major predictive factor of survival after curative surgical resection<sup>[93]</sup>.

Although there is no trial so far reporting results on the role of trastuzumab in the neoadjuvant setting, a number of case reports with trastuzumab-containing neoadjuvant chemotherapy regimens have been published, with interesting outcomes; complete pathological

responses were attained in 2 cases and a partial response with tumor mass reduction allowing for an extensive surgery in another case<sup>[94-96]</sup>.

### Pharmacokinetics and pharmacodynamics

Most data regarding the pharmacokinetic and pharmacodynamic profiles of trastuzumab stem from studies in breast cancer. A low systemic clearance ( $5.15 \pm 2.45$  mL/kg per day) and volume of distribution (44 mL/kg) have been described. Serum minimum concentrations of 10 µg/mL are needed to attain anti-proliferative effects and ADCC. With the usual loading dose of 4 mg/kg followed by 2 mg/kg per week, trastuzumab achieves and maintains serum minimum concentrations greater than 20 µg/mL. Recent results demonstrate that trastuzumab 6 mg/kg every 3 wk lead to the same plasma trough levels as trastuzumab 2 mg/kg weekly. Trastuzumab has been found not to exhibit dose-related nonlinear pharmacokinetics and the value of half-life of trastuzumab has an estimated value of 28.5 d<sup>[97,98]</sup>. No relevant drug interactions have been reported to date and elimination pathways remain largely unknown<sup>[99]</sup>. An extensive review about the pharmacodynamic and pharmacokinetic profiles, tolerability and dosage of trastuzumab in gastric cancer has been elaborated by Croxtall *et al*<sup>[100]</sup>. Targeted delivery systems involving anti-HER2 antibody mediated nano-scaled systems, drug conjugates, and fusion proteins are under active investigation<sup>[4,101,102]</sup>.

### Safety

The most commonly described adverse events with trastuzumab are infusion-related, described as fever, rigors, chills, nausea, dyspnea, and hypotension, and are present in about 40% of patients after the first administration and in 5% with subsequent treatment<sup>[103]</sup>. Trastuzumab has been extensively evaluated in breast cancer with a wide range of chemotherapeutic agents showing no significant overlapping toxicity, with one important exception, regarding an increased risk of cardiotoxicity. Trastuzumab-related cardiac dysfunction is largely reversible on withdrawal of the antibody. However, significant cardiopathy such as valvular heart disease, angina pectoris, previous transmural infarction and heart failure with left ventricular ejection fraction (LVEF) < 50% are generally regarded as counter-indications for trastuzumab use<sup>[28]</sup>. With the chemotherapy doublet regimen evaluated in the ToGA trial, trastuzumab contributed with little added toxicity; no increase in chemotherapy related grade 3-4 toxicities (68% both arms) or cardiac events (6% both arms) were found. Nonetheless the number of patients with cardiac dysfunction (considered a  $\geq$  10% drop in LVEF to an absolute value < 50%) was low in both arms (5% trastuzumab + chemotherapy *vs* 1% chemotherapy alone). The European Society for Medical Oncology<sup>[104]</sup>, issued a statement regarding the cardiac monitoring of patients receiving trastuzumab. Clinical evaluation and assessment of cardiovascular risk factors and comorbidities should be performed in every patient proposed for treatment with trastuzumab<sup>[105]</sup>. While screening algorithms for trastuzumab-induced cardiomyopathy provide guidance, patient-based strategies of surveillance remain important. Many clinical trials involving patients with metastatic breast cancer include a screening study to document the baseline LVEF, followed by serial monitoring at 8- to 16-wk intervals<sup>[106]</sup>.

In the ToGA trial, serious adverse events were reported in 32% of patients treated with trastuzumab + chemotherapy and 28% in the chemotherapy group; with treatment-related mortality of 3% and 1% respectively. The adverse events were similar between both groups, with no difference in the overall rate of adverse events. Nausea, neutropenia, vomiting, and anorexia were the most frequently reported adverse events. Patients treated with trastuzumab + chemotherapy had slightly higher rates of diarrhoea, stomatitis, anemia, thrombocytopenia, fatigue, chills, weight loss, pyrexia, mucosal inflammation, and nasopharyngitis<sup>[73]</sup>. In a phase II study with trastuzumab and cisplatin as first-line therapy in GEJ and gastric cancer, trastuzumab showed a favourable toxicity profile<sup>[69]</sup>.

### Resistance to trastuzumab

Whilst data regarding mechanisms of resistance to trastuzumab in gastroesophageal cancer is scarce, important information can be retrieved from previous knowledge in the treatment of breast cancer. Primary resistance to single-agent trastuzumab in HER2-overexpressing

metastatic breast carcinomas is described in 66%-88% of cases, with resistance eventually ensuing after a relatively short treatment period; in fact, the majority of patients who achieve an initial response to trastuzumab-based regimens develop resistance within 1 year (PFS between 6.7 and 7.4 mo)<sup>[85,107-109]</sup>.

Proposed resistance mechanisms include aberrations in the PI3K/AKT/mTOR pathway with or without loss of the phosphatase and tensin homologue protein (PTEN) tumor suppressor gene, accumulation of truncated forms of the HER2 receptor that lack the extracellular trastuzumab-binding domain (collectively known as p95HER2), loss of phosphatase, activation of other tyrosine kinase receptors including the insulin-like growth factor receptor (IGF-1R), increased expression of membrane-associated glycoprotein (MUC1 and MUC4) and cyclin E overexpression<sup>[85,109-111]</sup>.

PTEN inhibits PI3K, thereby inhibiting the PI3K/AKT/mTOR pathway. Loss of this tumor suppressor gene leads to at least partial resistance to trastuzumab. Indeed, both PIK3 mutations and PTEN loss were associated with inferior PFS and OS in a retrospective study of 256 HER2-positive metastatic breast cancer patients treated with trastuzumab<sup>[112]</sup>. A potential role for PI3K, AKT or mTOR inhibitors seems to exist, since these agents preclude the constitutive activation of this pathway, reversing PTEN loss-induced trastuzumab resistance<sup>[113-116]</sup>.

Truncated forms of HER2 which arise through the proteolytic shedding of the extracellular domain of full-length HER2 or by alternative translation initiation from two methionine residues are the predominant HER2 forms in some tumors. The biological function of p95HER2 has not been fully characterized, though overexpression of p95HER2 has been shown to lead to growth of tumor xenografts in nude mice. The p95HER2 protein has kinase activity, and this activity is required for tumor growth; however, the mechanisms involved and its possible relationship with those used by full-length HER2 are still unknown. Importantly, since p95HER2 lacks the binding site for trastuzumab, it conveys resistance to this antibody. p95HER2 is expressed in approximately 30% of HER2-positive breast tumors and is correlated with poor disease-free survival and increased nodal metastasis when compared with patients that express full-length HER2<sup>[110,117]</sup>. p95HER2 can therefore be seen as a prognostic and predictive biomarker in breast cancer. In one study analysing 93 metastatic breast tumors, patients overexpressing p95HER2 were found to have a higher incidence of lung metastases and had significantly shorter PFS and OS with trastuzumab treatment in comparison with patients expressing only the full-length receptor<sup>[118]</sup>. Tumors that express p95HER2 may be resistant to trastuzumab but sensitive to the inhibitory effects of lapatinib, a low-molecular-weight dual tyrosine kinase inhibitor (TKI) of HER1/2 that has activity in patients with HER2-expressing tumors that are resistant to trastuzumab. Combination of trastuzumab

with lapatinib has been evaluated in women with HER2-positive, trastuzumab-refractory metastatic breast cancer. Lapatinib with trastuzumab was superior to lapatinib alone in clinical benefit: complete response, partial response, and stable disease for  $\geq 24$  wk was observed in 24.7% of patients in the combination arm *vs* 12.4% in the monotherapy arm<sup>[119,120]</sup>. According to some authors this combination could provide a chemotherapy-free option after first line chemotherapy + trastuzumab<sup>[109]</sup>.

Increased signalling through other receptor TKIs including EGFR, HER3, MET and IGF-1R has been found in cells resistant to HER2-targeting treatments<sup>[109]</sup>. PI3K/AKT/mTOR pathway activation through up-regulation of HER3 signalling was demonstrated after exposure of breast cancer cells to HER TKIs<sup>[121]</sup>. On the other hand, pertuzumab, a HER2-HER3 dimerization inhibitor has demonstrated activity against trastuzumab resistant breast cancer cells<sup>[122]</sup>. Taking this findings into account, HER3 seems to play an important role in the mechanism of trastuzumab resistance.

In preclinical studies, co-expression of HER2 and IGF-1R in breast cancer cells resulted in loss of sensitivity to trastuzumab, conversely, inhibiting ligand-mediated activation of IGF-1R restored sensitivity to trastuzumab, therefore pointing towards a possible strategy to reduce or delay trastuzumab resistance<sup>[123,124]</sup>.

### Overcoming resistance to trastuzumab

Strategies to overcome trastuzumab resistance imply the important fact that many HER2-positive gastric tumors retain dependency on downstream signalling *via* the HER2 pathway. Therefore, besides other anti-HER2 agents (described in the following section), a focus on targeting these downstream signalling molecules has emerged<sup>[125,126]</sup>. Implied targets include mTOR inhibitors, HSP90 inhibitors and MET inhibitors; particularly interesting data exists concerning the possibility to combine some of these agents with anti-HER2 agents on which a patient has progressed, as the potential to reverse resistance to trastuzumab has been demonstrated<sup>[127-129]</sup>.

## OTHER ANTI-HER2 AGENTS

### Lapatinib

Lapatinib is a dual TKI active on both EGFR and HER2, with known activity in trastuzumab resistant advanced breast cancer; data suggests that there is no cross-resistance with trastuzumab and lapatinib restored trastuzumab sensitivity in preclinical models<sup>[28,130,131]</sup>. Wainberg *et al*<sup>[132]</sup> evaluated the effect of lapatinib in HER2-amplified cell lines and xenograft models, concluding that lapatinib inhibits the growth of HER2-amplified cancer cell lines, induces cell cycle arrest and apoptosis and acts synergistically with trastuzumab.

It is approved as combination therapy with capecitabine for patients with HER2-overexpressing breast cancer with prior progression on trastuzumab, an anthracycline and a taxane<sup>[133]</sup>. In a phase II trial conducted by Galsky

*et al*<sup>[134]</sup>, patients with HER2 amplified gastro-esophageal, bladder, ovarian, or uterine tumors were enrolled into a double-blinded randomized discontinuation study of lapatinib 1500 mg *per os* a day. Of a total of 141 patients screened, 32 patients with HER2 amplified tumors were enrolled in the study. At 3 mo, 1 (3%) patient had a complete response (CR), 9 (28%) had stable disease, 20 (63%) had progressive disease, and 2 (6%) were unknown. Unfortunately, due to low response rate and slow enrolment, the study had to be closed early. Concerning gastro-esophageal cancer, a modest CR rate of 6.25% was reported. A phase II study with lapatinib as first-line therapy in 47 patients with advanced gastric cancer showed modest single-agent activity, with 12% response rate, 20% disease stabilization, 7% of patients experiencing partial response and a median OS of 5 mo, less than that seen with conventional cytotoxic chemotherapy<sup>[135]</sup>. Another phase II study of lapatinib monotherapy in patients with HER2-overexpressing GEJ or esophageal cancer reported limited single-agent activity, with no objective responses and stable disease in 8% of patients<sup>[136]</sup>. Lapatinib in conjunction with capecitabine in the first line treatment of HER2 positive metastatic gastric cancer setting was addressed in a multicenter phase II trial, reporting a response rate of 22% and stable disease rate of 45%<sup>[137]</sup>. In another phase II trial, partial response of 24% and stable disease in 34% of patients was reported with lapatinib + capecitabine. Most frequent grade 3 and 4 side effects were anorexia, hand-foot syndrome, anemia and nausea; no significant cardiotoxicity was reported<sup>[138]</sup>. Two phase III studies evaluating the role of lapatinib in combination with chemotherapy in advanced esophago-gastric cancer are currently being conducted, the LOGIC trial<sup>[139,140]</sup> (combination of lapatinib with oxaliplatin and capecitabine as first-line treatment) and the TYTAN trial<sup>[141,142]</sup> (lapatinib in combination with weekly paclitaxel in second-line setting). OS results from the LOGIC trial are expected in 2014. Data from the TYTAN trial were presented at ASCO GI 2013. 430 patients were randomized, with an OS of 11 mo for the experimental arm *vs* 8.9 mo for the paclitaxel-alone arm; the subgroup of patients with HER2 3+ expression score attained an OS of 14 mo.

As previously stated, dual blockade with lapatinib and trastuzumab in metastatic breast cancer patients that progressed on trastuzumab-containing regimens improved PFS and clinical response rate<sup>[120]</sup>; a clinical case reported durable stable disease in a patient treated with this strategy despite progression during prior chemotherapy with trastuzumab<sup>[143]</sup>.

### Pertuzumab

Pertuzumab is a monoclonal antibody targeting HER2 in domain II (Figure 1), preventing formation of the highly mitogenic HER2/HER3 dimer. Available data stem mostly from breast cancer. As with trastuzumab, the antibody is not effective in patients without amplification of HER2<sup>[144]</sup>. In the phase III CLEOPATRA

**Table 3 Clinical trials with other anti-human epidermal growth factor receptor 2 agents**

Setting, therapy line	ID	Phase	n	Treatment	Primary EP	Status
Operable disease	NCT00450203	III	370	Lapatinib, epirubicin, cisplatin, capecitabine	OS	Recruiting
Advanced first line	NCT00680901	III	535	Lapatinib, oxaliplatin, capecitabine	OS	Active, not recruiting
	LOGiC					
	NCT01395537	II	43	Lapatinib, carboplatin, paclitaxel	Safety, RR	Active, not recruiting
	NCT01123473	II	192	Lapatinib, epirubicin, cisplatin, capecitabine, 5-FU	PFS	Unknown
	NCT00526669	II	67	Lapatinib, capecitabine	RR	Active, not recruiting
Advanced second line	NCT00486954	III	273	Lapatinib, paclitaxel	OS	Completed
	TYTAN					
	NCT01522768	II	27	Afatinib	RR	Recruiting
	NCT01152853	II	28	Dacomitinib	PFS	Unknown
	NCT01145404	II	76	Lapatinib, capecitabine	RR	Active, not recruiting

OS: Overall survival; PFS: Progression-free survival; 5-FU: 5-fluorouracil; RR: Response rate; CR: Complete response; PR: Partial response; NA: Not available.

study, 808 patients with HER2-positive metastatic breast cancer received placebo + trastuzumab + docetaxel (control group) or pertuzumab + trastuzumab + docetaxel (pertuzumab group). Median PFS was 12.4 mo in the control group *vs* 18.5 mo in the pertuzumab group. The hazard ratio for the addition of pertuzumab to docetaxel + trastuzumab for PFS was 0.62, with moderate toxicity added by the second antibody<sup>[145]</sup>. Pre-clinical results show potentiation of trastuzumab antitumour activity when combined with pertuzumab<sup>[146]</sup>. Pertuzumab is currently under investigation in a phase II study, in the first line gastric setting in combination with trastuzumab and platinum-fluoropyrimidine based chemotherapy<sup>[147]</sup>.

### Trastuzumab emtansine

Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate, which combines trastuzumab with the targeted delivery of the cytotoxic agent DM1, a derivative of maytansine, and a potent antimicrotubule agent. As systemic therapy, gastrointestinal toxicity limits the therapeutic usefulness of the agent<sup>[109]</sup>. In xenograft models, T-DM1 was found more effective than trastuzumab alone, with positive results independent of the tumor burden at therapy initiation, or preceding treatment with trastuzumab<sup>[101]</sup>. In a phase II study by Burris *et al*<sup>[148]</sup>, T-DM1 had robust single-agent activity in patients with heavily pre-treated, HER2-positive metastatic breast cancer, with a favourable toxicity profile. In breast cancer, the EMILIA trial assigned patients with HER2-positive advanced breast cancer, previously treated with trastuzumab and a taxane, to T-DM1 or lapatinib + capecitabine. Median PFS was 9.6 mo with T-DM1 *vs* 6.4 mo with lapatinib plus capecitabine; with an objective response rate of 43.6% for T-DM1<sup>[149]</sup>. Taken together, results from preclinical studies and breast cancer clinical trials point out T-DM1 as a promising agent to be evaluated in gastro-esophageal cancer. Currently, a phase II-III study is ongoing to evaluate T-DM1 *vs* taxane in patients with previously treated locally advanced or metastatic HER2+ gastric and GEJ cancer.

### Pan-HER TKIs

Irreversible small molecule pan-HER TKIs causes tumor

regression in HER2-overexpressing human gastric cancer xenograft models. They act by inhibition of HER family receptor phosphorylation and blocking of hetero-dimerization among them. Pre-clinical data reveal a synergistic effect with other molecular targeted agents (including trastuzumab) and chemotherapeutic agents. Currently investigated pan-HER TKIs include dacomitinib and afatinib<sup>[150,151]</sup>.

Selected ongoing clinical trials exploring other anti-HER2 agents can be found in Table 3.

## OTHER HER2-DIRECTED STRATEGIES

HER2 vaccines, both DNA and peptide-based, are actively researched in the field of breast cancer and results indicate a possible future role for this modality in combination with other HER2 targeted therapies. A phase I study carried out by Hamilton *et al*<sup>[152]</sup> combined HER2 immunization with lapatinib found this combination to be safe and immunogenic, however, the anticancer activity of immunization-induced antibodies is still not well characterized. Successful repression of the HER2 gene by the means of adenovirus constructs rises expectations for possible applications in cancer treatment<sup>[153]</sup>. Radioimmunotherapy is another possible application of HER2 directed homing, with authors currently evaluating 212Pb immunoconjugates with trastuzumab in intraperitoneal application after preclinical studies showed interesting results<sup>[154,155]</sup>.

## CONCLUSION

Now, times are changing. New strategies had been developed and implemented for advanced gastric cancer treatment. HER2 acquired a main role in gastric cancer management and current is also mandatory in order to predict trastuzumab response in association with standard platinum-based chemotherapy. Furthermore, others drugs are in developing to overcome resistance to trastuzumab, serious treatment-related toxicities and also help oncologists to improve treatments approaches. In future, genomic profiles will probably be part of clinical routines

for personalizing therapies and improve outcomes of those patients.

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## New insights in bilirubin metabolism and their clinical implications

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### Abstract

Bilirubin, a major end product of heme breakdown, is an important constituent of bile, responsible for its characteristic colour. Over recent decades, our understanding of bilirubin metabolism has expanded along with the processes of elimination of other endogenous and exogenous anionic substrates, mediated by the action of multiple transport systems at the sinusoidal and canalicular membrane of hepatocytes. Several inherited disorders characterised by impaired bilirubin conjugation (Crigler-Najjar syndrome type I and type II, Gilbert syndrome) or transport (Dubin-Johnson and Rotor syndrome) result in various degrees of hyperbilirubinemia of either the predominantly unconjugated or predominantly conjugated type. Moreover, disrupted regulation of hepatobiliary transport systems can explain jaundice in many acquired liver disorders. In this review, we discuss the recent data on liver bilirubin handling based on the discovery of the molecular basis of Rotor syndrome. The data show that a substantial fraction of bilirubin conjugates is primarily secreted by

MRP3 at the sinusoidal membrane into the blood, from where they are subsequently reuptaken by sinusoidal membrane-bound organic anion transporting polypeptides OATP1B1 and OATP1B3. OATP1B proteins are also responsible for liver clearance of bilirubin conjugated in splanchnic organs, such as the intestine and kidney, and for a number of endogenous compounds, xenobiotics and drugs. Absence of one or both OATP1B proteins thus may have serious impact on toxicity of commonly used drugs cleared by this system such as statins, sartans, methotrexate or rifampicin. The liver-blood cycling of conjugated bilirubin is impaired in cholestatic and parenchymal liver diseases and this impairment most likely contributes to jaundice accompanying these disorders.

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**Key words:** Hyperbilirubinemia; Hereditary jaundice; UGT1A1; ABCC2; Organic anion transporting polypeptide 1B1; Organic anion transporting polypeptide 1B3

**Core tip:** Experiments with *Oatp1a/1b*-null mice and *Oatp1a/1b*; *Abcc3* combination knockout mice plainly demonstrated that even under physiologic conditions a substantial portion of bilirubin glucuronides is not excreted directly into bile but is transported back to the blood by *Abcc3*. *Oatp1a/1b* activity accentuated in downstream (centrizonal) hepatocytes allows efficient reuptake of bilirubin conjugates, with a subsequent possibility being safely eliminated by excretion into bile. This and molecular findings in Rotor syndrome suggest that human transporters MRP3 and OATP1Bs form a sinusoidal liver-to-blood cycle which mediates shifting (hopping) of bilirubin and other substrates from periportal to centrizonal hepatocytes (References 18, 19, 22, 125).

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## INTRODUCTION

Bilirubin is the end product of heme breakdown. About 80% of bilirubin originates from degradation of erythrocyte haemoglobin in the reticuloendothelial system; the remaining 20% comes from inefficient erythropoiesis in bone marrow and degradation of other heme proteins<sup>[1-4]</sup>. Water insoluble, unconjugated bilirubin (UCB) bound to albumin is transported to the liver where it is removed from the plasma. The exact mechanism of UCB uptake is unknown; however, passive transmembrane diffusion seems to be combined with active transport mediated by several sinusoidal transporters (see below). Within the cytoplasm of hepatocytes, bilirubin is bound to ligandin and transported to endoplasmic reticulum where conjugation with glucuronic acid takes place. Conjugation is catalysed by the enzyme uridine diphosphate glycosyltransferase 1A1 (UGT1A1; EC2.4.1.17), a member of an enzyme family in the endoplasmic reticulum and nuclear envelope of hepatocytes<sup>[5-8]</sup>. In addition to the liver, UGT activity has also been detected in the small intestine and kidney<sup>[9,10]</sup>. *UGT1A1* gene (ID: 54658) is a part of a complex locus encoding 13 UDP-glucuronosyltransferases<sup>[11]</sup>. The locus contains a series of thirteen unique alternate promoters and first exons, followed by four common exons No. 2-5. Theoretically, each of the unique first exons is spliced to the first of the four shared exons. The unique first exons encode different substrate binding domains whereas the other functional domains encoded by the shared exons 2-5 are the same<sup>[11-15]</sup>. In reality, only 9 of the 13 predicted *UGT1As* are active genes encoding functional enzymes; four are nonfunctional pseudogenes.

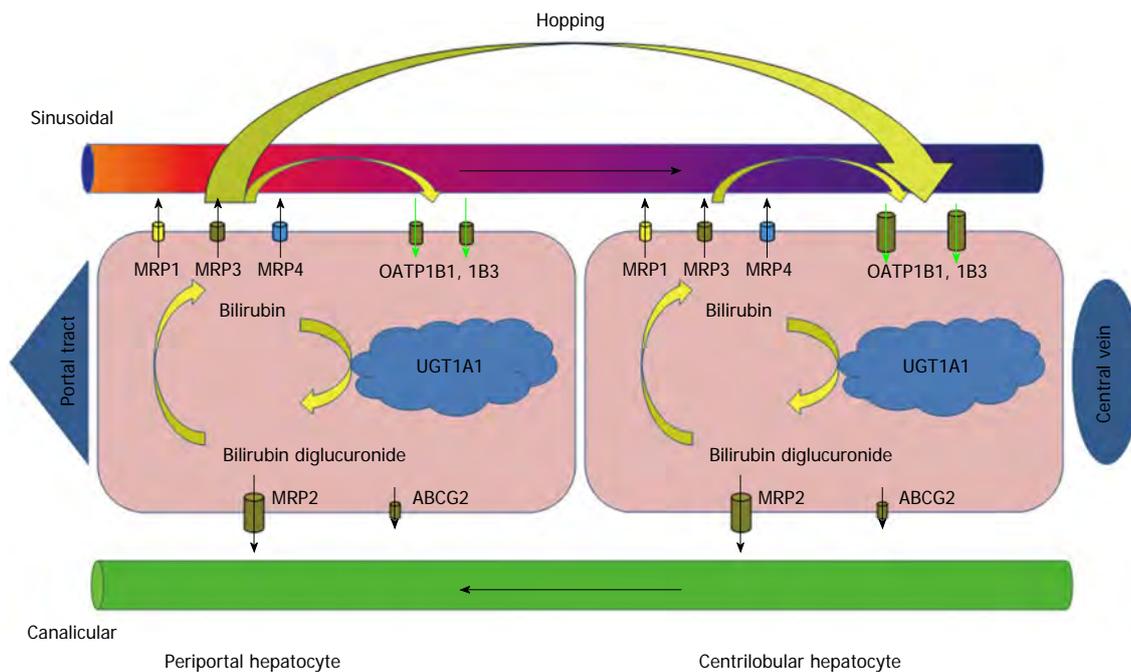
The excretion of conjugated bilirubin into bile is mediated by an ATP-dependent transporter identified as the multidrug resistance-associated protein MRP2/cMOAT and, to a lesser extent, also by ATP-binding cassette (ABC) efflux transporter ABCG2. MRP2 is encoded by *ABCC2* and expressed under physiologic conditions at the apical (canalicular) membrane of hepatocytes and, to a much lesser extent, in the kidney, duodenum, ileum, brain and placenta<sup>[16]</sup>. Since the MRP2 mediated export represents an important step in detoxification of many endogenous and exogenous substrates, the absence of functionally active MRP2 prevents the secretion of these conjugates into bile. Absence of MRP2 mediated transport is followed by upregulation of the basolateral MRP2 homologues at the sinusoidal membrane of hepatocytes and conjugated bilirubin flow is redirected into sinusoidal blood<sup>[17]</sup>. Aside from MRP2 mediated transport of conjugated bilirubin into bile, recent studies have shown that a significant fraction of the bilirubin conjugated in the liver is, under physiologic conditions, secreted into sinusoidal blood and subsequently reuptaken by hepatocytes for fi-

nal biliary excretion<sup>[18,19]</sup>. The process is mediated by sinusoidal transporters MRP3 and organic anion-transporting polypeptides OATP1B1 and OATP1B3. OATP1B transporters facilitate sodium-independent uptake of numerous endogenous and exogenous substrates<sup>[20,21]</sup>. Since expression of OATP1Bs is higher in centrilobular hepatocytes, the MRP3-OATP1B1/3 loop is likely responsible for shifting (hopping) of conjugated bilirubin and other substrates from the periportal to the centrilobular zone of the liver lobule (Figure 1). Such intralobular substrate transfer may protect periportal hepatocytes against elevated concentrations of various xenobiotics<sup>[22]</sup>. In addition, the OATP1B proteins mediate hepatic clearance of bilirubin conjugated in splanchnic organs and may represent an important alternative pathway in enterohepatic circulation<sup>[18]</sup>.

OATP1Bs may also contribute to liver uptake of UCB since complete absence of both OATP1Bs in Rotor syndrome (RS, see below) is associated with elevated levels of UCB and single nucleotide polymorphisms in genes encoding OATP1B proteins have been shown to influence serum bilirubin level<sup>[23,24]</sup>. Furthermore, results of functional studies demonstrate that OATP1B3, but not OATP1B1, may play an important role in the carrier-mediated uptake of foetal UCB by the placental trophoblast and contribute to elimination of UCB across the placental barrier<sup>[25,26]</sup>.

Mild or moderately elevated serum bilirubin seems to be beneficial: Bilirubin is known as a strong antioxidant<sup>[27,28]</sup> and the protective effects of bilirubin on atherogenesis and cancerogenesis have been demonstrated in both *in vitro* and *in vivo* studies<sup>[29-33]</sup>. On the other hand, patients with profound unconjugated hyperbilirubinemia are at risk for bilirubin encephalopathy (kernicterus)<sup>[34,35]</sup>. The toxic effects of bilirubin are explained by inhibition of DNA synthesis<sup>[36]</sup>. Bilirubin may also uncouple oxidative phosphorylation and inhibit adenosine triphosphatase (ATPase) activity of brain mitochondria<sup>[37,38]</sup>. Bilirubin mediated inhibition of various enzyme systems, RNA synthesis and protein synthesis in the brain and liver, and/or alteration of carbohydrate metabolism in the brain can also contribute to its toxicity<sup>[39-43]</sup>. The accumulation of bilirubin in plasma and tissues results in characteristic yellow discoloration of tissues known as icterus or jaundice.

Inherited disorders of bilirubin excretory pathway played the key role in understanding the individual steps of the bilirubin excretory pathway. Disrupted regulation of hepatobiliary transport systems explained jaundice in many acquired liver disorders<sup>[44-48]</sup>. Additional information was obtained from a number of animal models of hereditary jaundice. These include the Gunn rat and *Ugt1(-/-)* mouse mimicking the Crigler-Najjar syndrome type I<sup>[49-51]</sup>, the Bolivian population of squirrel monkeys mimicking Gilbert syndrome (GS)<sup>[52,53]</sup> and mutant TR or GY (Groningen yellow) rats with organic anion excretion defect (TR *-/-*), Eizai hyperbilirubinuria rats (EHBR), mutant Corriedale sheep, and *Mrp2(-/-)* mice, all modelling the Dubin-Johnson syndrome (DJS)<sup>[54-58]</sup>.



**Figure 1** Liver cycle of conjugated bilirubin. Bilirubin conjugated in endoplasmic reticulum of hepatocytes is secreted into the bile. This process is mediated by MRP2/ABCC2 with possible minor contribution of other transporters (ABCG2) at the canalicular membrane of hepatocytes. In addition, even under physiologic conditions, a fraction of bilirubin conjugates is secreted by MRP3 across the sinusoidal membrane into the blood, from where they can be subsequently reuptaken by sinusoidal membrane-bound OATP1B1 and OATP1B3 transporters. The highest overall expression of OATP1Bs has been demonstrated at the centrilobular hepatocytes. The process of substrate shifting (hopping) from periportal to centrilobular hepatocytes may act as a protection of the periportal hepatocytes against elevated concentrations of various xenobiotics. MRP: Multidrug resistance-associated protein; OATP: Organic anion transporting polypeptide; UGT: Uridine diphosphate glucuronosyltransferase; ABC: ATP-binding cassette.

## HEREDITARY PREDOMINANTLY UNCONJUGATED HYPERBILIRUBINEMIA

Conjugation of bilirubin in endoplasmic reticulum is catalysed by the enzyme UGT1A1. Mutations in *UGT1A1* can lead to decreased expression or partial or even complete inactivation of the enzyme<sup>[59]</sup>. By contrast, expression of *UGT1A1* can be increased by phenobarbital (PB) administration. PB response activity is delineated to a 290-bp distal enhancer module sequence (-3483/-3194) glucuronosyltransferase phenobarbital response enhancing motif (gtBPREM) of the human *UGT1A1*<sup>[59,60]</sup>. gtBPREM is activated by the nuclear orphan receptor, human constitutive active receptor (hCAR). CAR is a cytoplasmic receptor which, after treatment with activators such as PB, translocates into the nucleus, forms a heterodimer with the retinoid X receptor and activates the PB response enhancer element.

Three types of inherited, predominantly unconjugated hyperbilirubinemia with different levels of UGT1A1 activity are recognised: Crigler-Najjar syndrome type I (CN1), type II (CN2) and GS.

CN1 (MIM#218800), the most deleterious form, described in 1952 by Crigler and Najjar<sup>[61]</sup>, is characterised by complete or almost complete absence of UGT1A1 enzyme activity with severe jaundice<sup>[62]</sup>. Icterus occurring shortly after birth is complicated by bilirubin encephalopathy (kernicterus). Until the introduction of phototherapy and plasmapheresis, kernicterus was fatal in almost all cases during the first two years of life or caused seri-

ous brain damage with permanent neurologic sequelae. Intermittent phototherapy is lifelong and it results in a thorough elimination of water-soluble photoisomers of unconjugated bilirubin *via* bile. The effectiveness of phototherapy may decrease gradually with age and patients are at higher risk of sudden brain damage<sup>[63]</sup>.

Although new treatment modalities such as hepatocyte or hepatic progenitor cell transplantation have already been used to treat CN1 patients, liver transplantation is still considered to be the only definitive treatment for CN1<sup>[63-67]</sup>. Gene therapy seems to be a promising therapeutic possibility for the patients with CN1 in the near future<sup>[68,69]</sup>.

CN2 (Arias syndrome, MIM #606785), described by Arias in 1962<sup>[70]</sup>, is characterised by reduced UGT1A1 enzyme activity with a moderate degree of nonhemolytic jaundice. Bilirubin levels do not exceed 350  $\mu\text{mol/L}$  and CN2 is only rarely complicated by kernicterus<sup>[71]</sup>. Virtually all the mutations responsible for the syndrome are autosomal recessive, as in CN1, but several observations have also suggested the possibility of autosomal dominant pattern of inheritance<sup>[72-74]</sup>.

An important clinical difference between CN type I and type II is the response to PB treatment, with no effect in type I (complete loss of the UGT1A1 enzyme activity) and a decrease of serum bilirubin levels by more than 30% in CN type II (some residual UGT1A1 activity is preserved). Moreover, bilirubin glucuronides are present in bile in CN2. However, the method of choice for the diagnosis of CN syndrome is mutation analysis of

*UGT1A1*<sup>[75]</sup>.

GS (MIM #143500), described in 1901 by Gilbert and Lereboullet<sup>[76]</sup>, is characterised by fluctuating mild, unconjugated nonhemolytic hyperbilirubinemia < 85 µmol/L without overt haemolysis, usually diagnosed around puberty, and aggravated by intercurrent illness, stress, fasting or after administration of certain drugs<sup>[77,78]</sup>. Physical examination and the results of routine laboratory tests are normal apart from elevated serum bilirubin and jaundice. The clinical diagnosis of GS can be established if patients have a mild, predominantly unconjugated hyperbilirubinemia and normal activity of liver enzymes. The reduced caloric intake test and phenobarbital stimulation test have low diagnostic specificity in GS subjects<sup>[79]</sup>. Histological findings in GS are mild, with a slight centrilobular accumulation of pigment with lipofuscin-like properties<sup>[80]</sup>. Ultrastructurally, hepatocytes reveal hypertrophy of smooth endoplasmic reticulum<sup>[81,82]</sup>. Since the morphological picture of GS is completely non-specific and the disorder is benign, liver biopsy is not indicated.

GS is characterised by reduced levels of *UGT1A1* activity to about 25%-30% caused by homozygous, compound heterozygous, or heterozygous mutations in the *UGT1A1* with autosomal recessive transmission<sup>[80]</sup>.

GS is the most frequent hereditary jaundice affecting nearly 5%-10% of the Caucasian population<sup>[83]</sup>. The genetic basis of GS was first disclosed in 1995<sup>[84]</sup> as presence of the allele *UGT1A1\*28*, characterised by insertion of TA in the TATAA box (A[TA]<sub>n</sub>TAA) in the proximal promoter of *UGT1A1*. *UGT1A1\*28* has been identified as the most frequent mutation in Caucasian GS subjects<sup>[85]</sup>. The insertion is responsible for reduction of transcription of *UGT1A1* to 20% from normal and for a decrease of hepatic glucuronidation activity by 80% in a homozygous state<sup>[86]</sup>. In Caucasians and African Americans, the frequency of *UGT1A1\*28* allele is about 35%-40%, but it is much lower in Asians, including Koreans (13%), Chinese (16%), and Japanese (11%)<sup>[87-89]</sup>. Moreover, in the majority of Caucasian GS subjects, expression of *UGT1A1* is further decreased by the presence of the second mutation T>G in *gtPBREM*<sup>[59,60]</sup>. In addition to the mutations in the promoter, GS may be caused by mutations in structural regions of the *UGT1A1*. In Asians, other variants, such as *UGT1A1\*6* characterised by a missense mutation involving G to A substitution at nucleotide 211 (c.211G>A) in exon 1 (also known as p.G71R), *UGT1A1\*7* (p.Y486D), *UGT1A1\*27* (p.P229Q), and *UGT1A1\*62* (p.F83L) have been detected<sup>[60,87-90]</sup>.

In addition to biochemical defect leading to reduced glucuronidation, other factors, such as impaired hepatic (re)uptake of bilirubin (see Rotor syndrome below for the possible mechanism) or an increased load of bilirubin, seem to be necessary for clinical manifestation of GS<sup>[86,91,92]</sup>.

GS is benign and GS carriers present with no liver disease. However, the mutations in the *UGT1A1* identical to those recognised in GS subjects may contribute to the

development of prolonged neonatal hyperbilirubinemia in breast-fed infants<sup>[93,94]</sup>.

Moreover, since the process of glucuronidation is an important step in elimination of numerous endogenous and exogenous substrates, GS subjects may be more susceptible to the adverse effects of some drugs metabolised by *UGT1A1*, such as indinavir, atazanavir<sup>[95-99]</sup> or irinotecan<sup>[100-102]</sup>.

## HEREDITARY PREDOMINANTLY CONJUGATED HYPERBILIRUBINEMIA

Two types of hereditary conjugated jaundice are known as Dubin-Johnson and Rotor syndrome. Both are characterised by the presence of mixed, predominantly conjugated hyperbilirubinemia, with conjugated bilirubin more than 50% of total bilirubin.

DJS (MIM # 237500), a benign autosomal recessive disorder described in 1954 by Dubin *et al*<sup>[103]</sup> and Sprinz *et al*<sup>[104]</sup>, is characterised by fluctuating mild, predominantly conjugated hyperbilirubinemia, with typical manifestation in adolescence or young adulthood. Most patients are asymptomatic except of occasional slight abdominal pain and fatigue. Urine excretion of total coproporphyrin in 24 h is normal, but 80% are represented by coproporphyrin I. Biliary excretion of anionic dyes including bromosulfophthalein (BSP), indocyanine green and cholescintigraphy radiotracers is delayed with absent or delayed filling of the gallbladder<sup>[105]</sup>. BSP clearance in DJS subjects is normal at 45 min with the second peak at 90 min<sup>[106]</sup>. Liver histology in DJS shows an accumulation of distinctive melanin-like lysosomal pigment in an otherwise normal liver that gives the organ a characteristic dark pink or even black colour. The pigment is positive in PAS and Masson-Fontana reaction with marked autofluorescence. In contrast to melanin, DJS pigment does not reduce neutral silver ammonium solution<sup>[103,107]</sup>. The amount of pigment may vary and possible transient loss may occur in coincidence with other liver diseases<sup>[108,109]</sup>. The molecular mechanism in DJS is absence or deficiency of human canalicular multispecific organic anion transporter MRP2/cMOAT caused by homozygous or compound heterozygous mutation in *ABCC2* (gene ID: 1244) on chromosome 10q24<sup>[110-114]</sup>. The *ABCC2* mutation alters not only MRP2-mediated transport of conjugated bilirubin but also transport of many anionic substrates as well as a wide range of drugs, such as chemotherapeutics, uricosurics, antibiotics, leukotrienes, glutathione, toxins and heavy metals. Absence of MRP2/cMOAT may result in impaired elimination and in subsequent renal toxicity of the substrates mentioned above<sup>[115-120]</sup>.

A rare type of hereditary mixed hyperbilirubinemia caused by the simultaneous presence of mutations characteristic for DJS and GS has been classified as dual hereditary jaundice<sup>[121]</sup>. Serum direct bilirubin concentrations in dual hereditary jaundice reach only 20%-50% of total bilirubin.

RS (MIM #237450), described in 1948 by Rotor *et al*<sup>[122]</sup>,

is characterised by mild, predominantly conjugated hyperbilirubinemia with delayed excretion of anionic dyes without re-increase of their concentration. Total urinary coproporphyrin excretion is significantly increased and the proportion of coproporphyrin I in urine is approximately 65% of the total in homozygotes and 43% in heterozygotes<sup>[123,124]</sup>. By histopathological examination, the liver tissue does not display any marked architectural or cytomorphological abnormalities and pigment is not present.

The presence of homozygous mutations in both *SLCO1B1* and *SLCO1B3* neighbouring genes located on chromosome 12 with complete and simultaneous deficiency of proteins OATP1B1 and OATP1B3 has recently been identified as the molecular mechanism of the syndrome<sup>[125]</sup>. The complete absence of both transporters OATP1B1 and OATP1B3 has been confirmed by immunohistochemistry in all studied Rotor subjects. Interestingly, the presence of a single functional allele of either *SLCO1B1* or *SLCO1B3* prevented the jaundice.

RS does not require any therapy but, with regard to the impact of OATP1B transporters on pharmacokinetics of a broad spectrum of commonly used drugs such as penicillins, statins, sartans, rifampicin, methotrexate and many others, it is assumed that RS subjects and also those with the deleterious mutations in either of the *SLCO1B* genes, even without full clinical expression of the syndrome, may be at increased risk for drug toxicity<sup>[125-129]</sup>.

## BILIRUBIN HANDLING PROTEINS IN CHOLESTASIS

Animal models of obstructive and intrahepatic cholestasis help us to discover and understand the main principles of acquired defects in hepatobiliary transport of bile salts and other organic anions. Up and down regulation of these mechanisms can explain impaired liver uptake and excretion of the biliary constituents resulting in the cholestasis and icterus which accompanies many common acquired liver disorders<sup>[48,130,131]</sup>. A general pattern of response to cholestatic liver injury is initiated by down-regulation of the basolateral membrane bound transporters NTCP and OATP1B1. The expression of several canalicular export pumps is relatively unaffected [bile salt export pump (BSEP), multidrug resistance protein 2 (MDR2)] or even upregulated (MDR1). Decreased expression of MRP2 in sepsis or in obstructive cholestasis is followed by upregulation of several MRP homologues at basolateral membrane of hepatocytes that may extrude bile salts back to the sinusoidal blood and systemic circulation. Most of these changes are believed to help prevent an accumulation of potentially toxic bile components and other substrates in the liver.

Similar patterns of expression of the bilirubin and bile salts handling proteins and mRNA are observed in cholestatic liver diseases in humans. At the stage I and II of primary biliary cirrhosis (PBC), expression and localisation of OATP1B1, OATP1B3, NTCP, MRP2, MRP3

and MDR3 are unchanged. At stage III, immunostaining intensities of the sinusoidal uptake transporters and their mRNA levels decrease. Irregular MRP2 immunostaining suggests redistribution of MRP2 into intracellular structures in the advanced stages of PBC; however, at stage III and IV, basolateral uptake transporters NTCP and OATP1B1 are downregulated. Expression of the canalicular export pumps for bile salts (BSEP) and bilirubin (MRP2) remains unchanged and the canalicular P-glycoproteins MDR1 and MDR3 and the basolateral efflux pump MRP3 are upregulated<sup>[132-135]</sup>.

At the early-stages of cholestasis in extrahepatic biliary atresia, BSEP, MDR3, MRP2, NTCP/SLC10A1, SLCO1A2 and nuclear receptor farnesoid X receptor are downregulated. At the late-stages of cholestasis, farnesoid X receptor and BSEP levels returns to normal, MDR3 and MDR1 are upregulated and MRP2 is downregulated<sup>[136]</sup>.

In primary sclerosing cholangitis, the level of OATP1B1 mRNA in liver tissue has been demonstrated to represent 49% of controls and the level of MRP2 mRNA dropped to 27% of controls<sup>[137]</sup>.

## CONCLUSION AND PERSPECTIVES

Over the last decades, molecular basis of hyperbilirubinemia syndromes has been elucidated and mutations affecting the basolateral and apical membrane transporters responsible for accumulation of either conjugated or unconjugated bilirubin have been identified.

Except for GS, the majority of inherited hyperbilirubinemia syndromes are rare autosomal recessive disorders with a low prevalence in the general population and, apart from CN syndrome type I and some cases of CN type II in neonatal period, mostly not requiring further therapy. Nonetheless, the enzyme and transport systems involved in bilirubin metabolism may play an important role in the elimination and disposition processes of many other endogenous and exogenous substrates including hormones, drugs, toxins and heavy metals<sup>[102,138]</sup>. Dysfunction or absence of these systems, including selected ABC transporters and OATPs, may alter pharmacokinetics and pharmacodynamics of many biologically active agents, affect penetration of the substrates into various tissues and lead to their intracellular accumulation with a subsequent increase of organ toxicity<sup>[126,127,128]</sup>. In addition, the absence of the functional transport proteins involved in hepatobiliary and enterohepatic circulation may involve drug disposition, drug-drug or drug-food interactions and result in decreased effectiveness or even resistance to a diverse spectrum of chemotherapeutic agents and xenobiotics<sup>[139-141]</sup>. Individuals with mutations in the responsible gene or genes with the fully expressed phenotype of the corresponding hyperbilirubinemia syndrome, as well as subjects carrying mutations without clinical manifestation of hyperbilirubinemia under normal conditions, may be more susceptible to the adverse effects of some drugs and metabolites<sup>[142,143]</sup>.

Clarifying the molecular genetic basis of hereditary hyperbilirubinemia syndromes together with the discoveries of the major systems essential for the metabolism and transport of bilirubin and other endogenous and exogenous substrates represent a substantial contribution to the current knowledge of the heme degradation pathway. Further investigation of how bilirubin transport proteins and their variations affect pharmacokinetics of drugs may be of significant clinical importance.

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## Irritable bowel syndrome and organic diseases: A comparative analysis of esophageal motility

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### Abstract

**AIM:** To assess the esophageal motility in patients with irritable bowel syndrome (IBS) and to compare those with patients with autoimmune disorders.

**METHODS:** 15 patients with IBS, 22 with systemic lupus erythematosus (SLE) and 19 with systemic sclerosis (SSc) were prospectively selected from a total of 115 patients at a single university centre and esophageal motility was analysed using standard manometry (Mui Scientific PIP-4-8SS). All patients underwent esophago-gastro-duodenoscopy before entering the study so that

only patients with normal endoscopic findings were included in the current study. All patients underwent a complete physical, blood biochemistry and urinary examination. The grade of dysphagia was determined for each patient in accordance to the intensity and frequency of the presented esophageal symptoms. Furthermore, disease activity scores (SLEDAI and modified Rodnan score) were obtained for patients with autoimmune diseases. Outcome parameter: A correlation coefficient was calculated between amplitudes, velocity and duration of the peristaltic waves throughout esophagus and patients' dysphagia for all three groups.

**RESULTS:** There was no statistical difference in the standard blood biochemistry and urinary analysis in all three groups. Patients with IBS showed similar pathologic dysphagia scores compared to patients with SLE and SSc. The mean value of dysphagia score was in IBS group 7.3, in SLE group 6.73 and in SSc group 7.56 with a  $P$ -value  $> 0.05$ . However, the manometric patterns were different. IBS patients showed during esophageal manometry peristaltic amplitudes at the proximal part of esophagus greater than 60 mmHg in 46% of the patients, which was significant higher in comparison to the SLE (11.8%) and SSc-Group (0%,  $P = 0.003$ ). Furthermore, IBS patients showed lower mean resting pressure of the distal esophagus sphincter (Lower esophageal sphincter, 22 mmHg) when compared with SLE (28 mmHg,  $P = 0.037$ ) and SSc (26 mmHg,  $P = 0.052$ ). 23.5% of patients with SLE showed amplitudes greater as 160 mmHg in the distal esophagus (IBS and SSc: 0%) whereas 29.4% amplitudes greater as 100 mmHg in the middle one (IBS: 16.7%, SSc: 5.9% respectively,  $P = 0.006$ ). Patients with SSc demonstrated, as expected, in almost half of the cases reduced peristalsis or even aperistalsis in the lower two thirds of the esophagus. SSc patients demonstrated a negative correlation coefficient between dysphagia score, amplitude and velocity of peristaltic activity at middle and lower esophagus [ $r = -0.6$ ,  $P <$

0.05].

**CONCLUSION:** IBS patients have comparable dysphagia-scores as patients with autoimmune disorders. The different manometric patterns might allow differentiating esophageal symptoms based on IBS from other organic diseases.

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**Key words:** Irritable bowel syndrome; Systemic lupus erythematosus; Systemic sclerosis; Esophageal manometry; Dysphagia

**Core tip:** This is the first comparative study concerning esophageal motility among functional and autoimmune disorders. Patients in irritable bowel syndrome (IBS)-group showed comparable dysphagia-scores as patients with systemic lupus erythematosus and systemic sclerosis. Nevertheless, different manometric patterns between the three examined groups were observed, which might allow differentiating esophageal symptoms based on IBS from other organic diseases.

Thomaidis T, Goetz M, Gregor SP, Hoffman A, Kouroumalis E, Moehler M, Galle PR, Schwarting A, Kiesslich R. Irritable bowel syndrome and organic diseases: A comparative analysis of esophageal motility. *World J Gastroenterol* 2013; 19(38): 6408-6415 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6408.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6408>

## INTRODUCTION

Irritable bowel syndrome (IBS) is a functional disorder of the gastrointestinal tract characterized mainly by symptoms as diarrhoea, constipation and diffuse abdominal pain<sup>[1]</sup>. The prevalence of IBS in the western population varies between 15% and 20%<sup>[2-7]</sup> with an overall 2:1 female predominance<sup>[5]</sup>. In 2006 the Rome III criteria for diagnosis and classification of IBS were established<sup>[8]</sup>. According to these criteria IBS is defined as recurrent abdominal pain or discomfort associated with altered defecation.

Dyspepsia and dysphagia are commonly reported of IBS patients. However, there are no comparative data available dealing with esophagus motility in IBS patients compared to autoimmune disorders. Analyses of peristaltic changes of the esophagus in patients with IBS have led to controversial findings<sup>[9-13]</sup>. Reduced resting pressure and relaxation of the lower esophageal sphincter (LES)<sup>[9,11]</sup>, abnormal contractions up to 150 mmHg<sup>[10]</sup> as well as normal peristaltic motility in patients with IBS<sup>[12]</sup> have been described.

Systemic sclerosis (SSc) and systemic lupus erythematosus (SLE) show a variety of visceral manifestations. Skin, joints, lungs, nervous system and internal organs

can be affected. Dysfunction of esophagus motility is reported in about 70%-90% of patients with SSc<sup>[14-18]</sup>. In patients with SLE the percentage varies between 1.5% and 25%<sup>[19]</sup>. Aim of the present study was to assess the esophageal motility function in patients with IBS compared to organic diseases (SLE and SSc).

## MATERIALS AND METHODS

### Subjects

Patients were prospectively invited to participate at this single centre study. Patients were identified based on the database of our outpatient clinic of gastroenterology and rheumatology at the University Mainz. Patients with IBS, SLE and SSc were able to participate. The database showed 115 patients with one of the mentioned diseases. 74 patients were screened and 56 patients agreed to participate in the study and were included (15 patients with IBS, 22 with SLE and 19 with SSc).

All SLE and SSc patients met the criteria established by the American Rheumatism Association for autoimmune diseases, whereas the patients in IBS group were included on the basis of Rome III process<sup>[8,20,21]</sup>. Patients with uncertain diagnosis, with known other severe diseases of the upper gastrointestinal tract and pregnant and lactating women were excluded from the study. All patients gave their written consent. The study was approved by the local ethics committee of Rheinland-Pfalz (No. 837.432.09).

### Methods

Besides thorough history and physical examination, the following diagnostic methods were performed:

**Blood biochemistry analysis:** A complete blood biochemistry examination including blood count and biochemical analysis were performed in each patient. Furthermore, the following parameters were examined: Complement factors C3 and C4, ANA, ENA, dsDNA, CRP and ESR.

**Upper endoscopy:** All patients underwent esophago-gastro-duodenoscopy (EGD) before entering the study. Only patients with normal endoscopic findings were included in the current study.

**Esophageal manometry:** Esophageal manometry was performed after 8 h of fasting. All medications which potentially could affect the esophageal motility were paused 48 h before manometry.

The measurements were performed using a 60 cm long, 8 channel lumen catheter (Sierra Scientific Instruments, Germany) with 5 distal openings separated 1 cm vertically and 3 proximal openings distributed at 5 cm distance apart.

Each of the catheter lumens was perfused with distilled water at a rate of 1.36 mL/min. The catheter was connected to an infusion system (Mui Scientific, Canada) with attached pressure converters. The catheter was ini-

**Table 1** Demographic data of the three examined groups *n* (%)

IBS	SLE		SSc	
	Diarrhoea	Constipation	Diarrhoea + Constipation	
Total	8 (53.3)	4 (26.7)		
Male	3 (100)	0	0	2 (9.01)
Female	8 (66.7)	1 (8.3)	3 (25)	20 (90.9)
Age (yr)	42 ± 16	56 ± 15	34 ± 18	48 ± 10
				55 ± 9

IBS: Irritable bowel syndrome; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis.

tially inserted transnasally into the patient’s stomach. The patients remained in a sitting position during insertion of the catheter.

After the lumen reached the stomach, patients were brought to a horizontal position and then the pressure of the LES was measured by using the station and rapid pull-through technique<sup>[22,23]</sup>.

Subsequently, the catheter was slowly withdrawn at 1 cm intervals with wet (10 mL water) and dry swallows at each level, so that a complete analysis of esophagus motility could be obtained. Ineffective swallows were not included in detailed measurements of manometric parameters.

The resulting esophageal parameters were: position, length, resting pressure and relaxation of the LES und upper esophageal sphincter (UES), mean peristaltic pressure, simultan or retrograde contractions, duration and velocity of the peristaltic waves at the proximal, medial and distal third of esophagus.

**Questionnaires**

Patients were asked to complete the following questionnaires:

**SLEDAI:** The disease activity in patients of SLE was assessed *via* the SLEDAI-Index<sup>[24]</sup>.

**Rodnan score:** The disease activity in patients with SSc was assessed *via* the modified Rodnan score<sup>[25]</sup>.

**Dysphagia score:** The intensity and frequency of the esophageal symptoms was assessed as an accumulation score as described before<sup>[26]</sup>. Specifically all patients were evaluated for symptoms such as odynophagia, difficulty in swallowing, chest pain, dysphagia etc in relation to frequency, need for treatment and weight loss.

**Statistical analysis**

The statistical analysis was conducted using SPSS program (version 19.0). Data were analyzed using the Mann-Whitney *U*-test to compare group means. A *P*-value < 0.05 was considered to represent a significant difference. Sample size estimation: Distinct sample size estimation could not be performed because of lack of comparative data. However, we hypothesised that IBS patients have at least 30% different manometric outcome parameters

**Table 2** Disease activity scores in patients with systemic lupus erythematosus and systemic sclerosis *n* (%)

Activity	Modified Rodnan score (average score)			SLEDAI (average score)		
	No activity	0	7 (39)	No activity	0	4 (19)
Mild	7.2	10 (55)		Mild	3	10 (48)
Moderate	18	1 (6)		Moderate	7.4	5 (24)
Severe	-	-		Severe	11	2 (9)
Total	5.2	18 (100)		Total	4.2	21 (100)

SLEDAI-score: 0: No activity; 2-5: Mild activity; 6-9: Moderate activity; 10-12: Severe activity. Modified Rodnan score: 0: No activity; 1-14: Mild activity; > 14: Moderate-severe activity.

compared to patients with autoimmune disorders, which lead to a sample size of 15 patients per group (Power 80%).

**RESULTS**

**Lab analysis**

There was no statistical difference in the standard lab values in all three groups. Patients with SLE and SSc showed as expected higher incidence in expression of autoimmune antibodies such as ANA, ENA, dsDNA or altered complement concentrations.

**Patient’s characteristics**

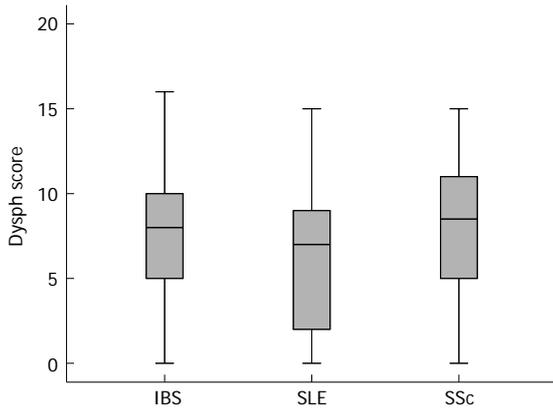
Patients with IBS showed higher prevalence of diarrhoea compared to constipation or to the combination of diarrhoea and constipation (Table 1). Patients with SLE and SSc reported a great variety of symptoms including weakness, difficulty in swallowing or non-specific musculoskeletal tenderness that can be explained due to secondary fibromyalgia. Gender and age showed no statistical significant changes within the three groups (Table 1).

**Disease activity in SLE and SSc**

The disease activity of SLE and SSc are shown in Table 2. SSc patients tend to have a milder disease activity compared to SLE patients. However, dysphagia score was similar in all three groups without any statistical difference. The mean value of dysphagia score was in IBS group 7.3, in SLE group 6.73 and in SSc group 7.56 with a *P*-value > 0.05 (Figure 1).

**Manometric analysis**

In the manometric studies we observed significant differences concerning the quality of the peristaltic waves (amplitude, duration and velocity) among the three groups (Figure 2). IBS patients showed increased peristalsis in the lower two thirds of esophagus in comparison to patients with SSc who in almost 50% of the cases manifested wide peristalsis with reduced amplitude and velocity (Figure 3). There was no significant difference between the amplitude, duration and velocity of the peristaltic movements in the lower two thirds of esophagus among



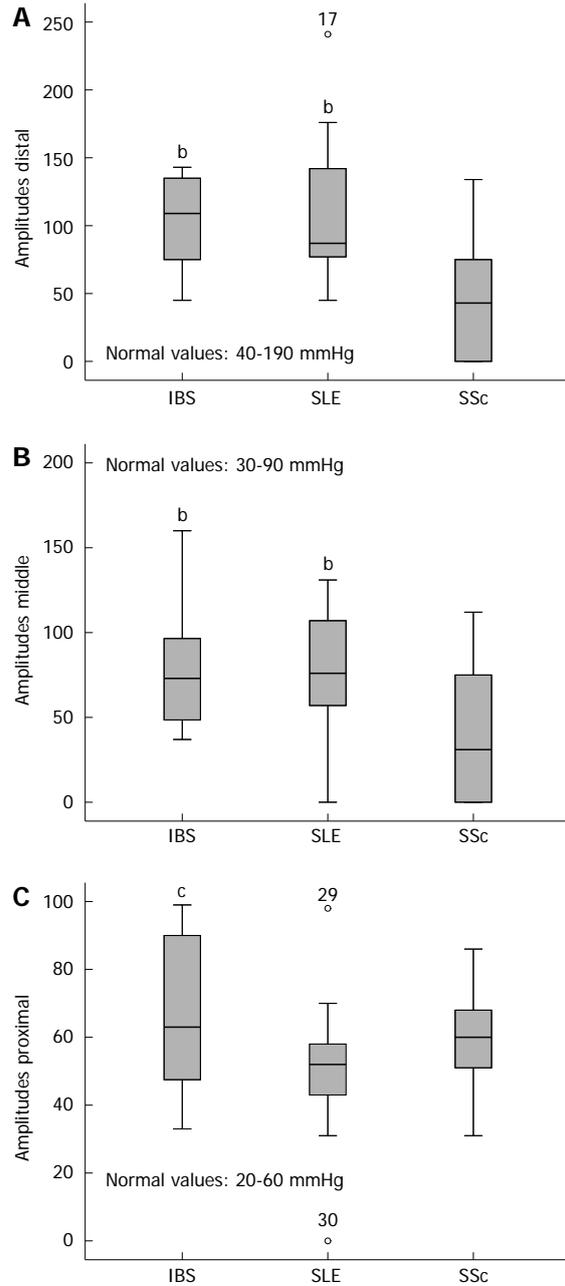
**Figure 1** Dysphagia score in patients with irritable bowel syndrome, systemic lupus erythematosus and systemic sclerosis. All groups showed similar abnormal dysphagia scores. The mean value of dysphagia score was in IBS group 7.3, in SLE group 6.73 and in SSc group 7.56. IBS: Irritable bowel syndrome; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis.

patients with IBS and patients with SLE. Nevertheless 23.5% of patients with SLE showed distal amplitudes greater as 160 mmHg and 29.4% middle amplitudes greater as 100 mmHg. At the proximal esophageal part patients with IBS showed significant higher peristaltic waves also when compared with patients in the SLE group (Table 3, Figure 2). Particularly, 45.5% of IBS patients showed amplitudes greater as 60 mmHg whereas in the SLE and SSc group the rates were 11.8% and 0% respectively (Figure 3). Measurements concerning the LES showed that patients with IBS had significant lower resting pressure in comparison to patients with autoimmune disorders (Table 3). Though, no significant difference could be observed when other manometric measures between the 3 groups were examined, such as length and relaxation of the lower esophageal sphincter as well as duration of distal and middle peristalsis. Regarding the UES we found a significant higher resting pressure and length by patients in IBS group in comparison to SLE and SSc (Table 3). Interestingly, 58.8% of patients with SLE and 56.35% of patients with SSc showed resting pressure less than 40 mmHg (Figure 3).

Correlation coefficient tests revealed a negative relation between dysphagia score, amplitude and velocity of peristaltic activity at middle and lower esophagus in SSc patients ( $r = -0,6, P < 0.05$ ). A connection between dysphagia and peristaltic abnormality in IBS and SLE groups was not observed. Furthermore, there was no association between the three subgroups of IBS, the score of dysphagia and manometric findings, as well as between the presence of autoantibodies and dysphagia among patients with SLE and SSc (data not shown).

## DISCUSSION

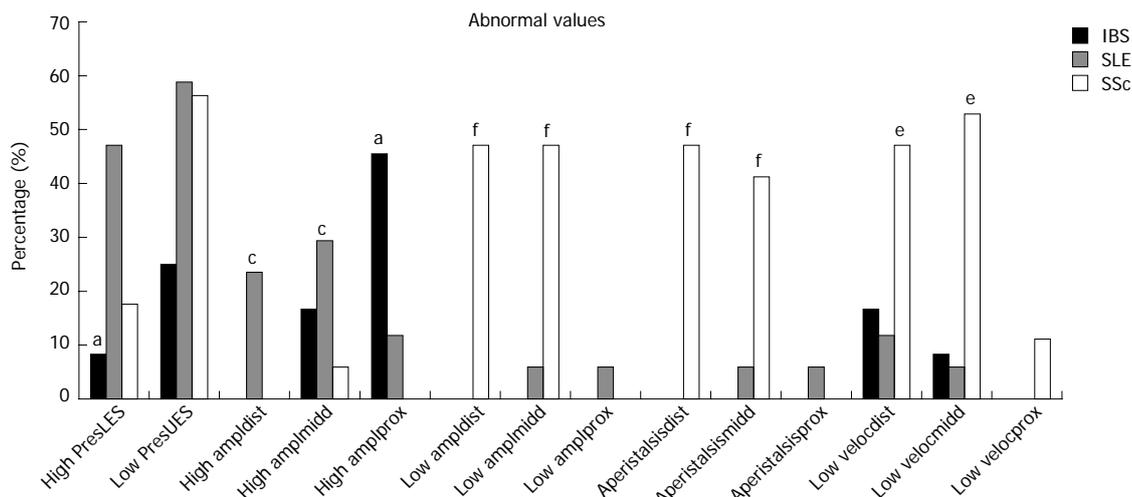
We conducted a comparative analysis of the peristalsis of the esophagus between patients with IBS and patients with SLE and SSc. The groups consisted in 66.7% IBS, 91% SLE and 84.2% SSc of female patients mainly be-



**Figure 2** Amplitudes of the peristaltic waves throughout esophagus. The mean value of amplitudes in patients with SSc was significant lower comparing to subjects with SLE and IBS in the middle (B) and distal (A) esophagus ( $P < 0.01$  vs SSc). In the proximal part (C) IBS patients showed higher peristaltic amplitudes in comparison to the other groups ( $P = 0.05$  vs SLE). Normal values as previously described. IBS: Irritable bowel syndrome; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis.

tween 40 and 60 years, which is completely in accordance with the epidemiology of the examined diseases<sup>[27-29]</sup>.

We found significant different manometric patterns in IBS patients compared to those with autoimmune disorders. It was interesting to notice that patients in the IBS group, as seen in the derived dysphagia scores, showed the same intensity and frequency of swallowing problematic as subjects in the two other categories. Specifically, patients with IBS complained about difficulties in swallowing, retrosternal sore and heartburn as frequent as pa-



**Figure 3** Comparative analysis of pathological manometric parameters in patients with irritable bowel syndrome, systemic lupus erythematosus and systemic sclerosis. 47.1% of SLE patients showed pressure of lower esophagus sphincter > 30 mmHg. 58.8% of SLE patients and 56.35% of SSc patients showed resting pressure of the upper esophageal sphincter < 40 mmHg. 23.5% of patients with SLE showed distal amplitudes > 160 mmHg and 29.4% middle amplitudes > 100 mmHg, whereas in the proximal part 45.5% of IBS patients had amplitudes > 60 mmHg. The majority of SSc patients showed as expected reduced peristaltic activity in the lower two thirds of esophagus. <sup>a</sup>*P* < 0.05 vs SLE and SSc; <sup>c</sup>*P* < 0.05 vs IBS and SSc; <sup>e</sup>*P* < 0.05, <sup>f</sup>*P* < 0.01 vs IBS and SLE. High PresLES: Pressure lower esophageal sphincter (LES) > 30 mmHg; Low PresUES: Pressure upper esophageal sphincter (UES) < 40 mmHg; High ampldist: Distal amplitude > 160 mmHg; High amplmidd: Middle amplitude > 100 mmHg; High amplprox: Proximal amplitude > 60 mmHg; Low ampldist: Distal amplitude < 50 mmHg; Low amplmidd: Middle amplitude < 40 mmHg; Low amplprox: Proximal amplitude < 30 mmHg; Low velocdist: Distal velocity < 2 cm/s; Low velocmidd: Middle velocity < 2 cm/s; Low velocprox: Proximal velocity < 2 cm/s. IBS: Irritable bowel syndrome; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis.

**Table 3** Comparative manometric findings in patients with with irritable bowel syndrome, systemic lupus erythematosus and systemic sclerosis

	IBS	SLE	SSc	<i>P</i> value
Lower esophageal sphincter				
Pressure (mmHg)	22 ± 5	28 ± 8.9	26 ± 5.2	<i>P</i> < 0.05 <sup>1,2</sup>
Length (cm)	3.6 ± 1.3	3.4 ± 1.1	3.6 ± 1	
Relaxation (%)	84.8 ± 15.4	89.5 ± 13.2	83.4 ± 10.4	
Distal esophagus				
Amplitude (mmHg)	103.6 ± 33.7	107.3 ± 53.3	43.3 ± 48.2	<i>P</i> < 0.001 <sup>2,3</sup>
Duration (s)	4.3 ± 1.4	4.7 ± 1.4	2.9 ± 2.9	
Velocity (cm/s)	3 ± 1.6	2.6 ± 1	1.4 ± 2	<i>P</i> < 0.05 <sup>2,3</sup>
Middle esophagus				
Amplitude (mmHg)	78.3 ± 35.7	75.9 ± 35	39.2 ± 42.4	<i>P</i> < 0.01 <sup>2,3</sup>
Duration (s)	3.3 ± 1.3	3.4 ± 1.3	2.3 ± 2.2	
Velocity (cm/s)	3.5 ± 1.4	3.9 ± 3.4	1.3 ± 3.7	<i>P</i> < 0.05 <sup>2,3</sup>
Proximal esophagus				
Amplitude (mmHg)	67.3 ± 23.3	50.6 ± 19.8	58.3 ± 15.9	<i>P</i> = 0.05 <sup>1</sup>
Duration (s)	2.5 ± 0.7	2.2 ± 0.7	2.8 ± 0.6	
Velocity (cm/s)	2.5 ± 1.1	3.6 ± 2.3	3.0 ± 2.4	
Upper esophageal sphincter				
Pressure (mmHg)	70.5 ± 22.3	52.7 ± 20	50.2 ± 17.8	<i>P</i> < 0.05 <sup>1,2</sup>
Length (cm)	3.6 ± 1.0	2.8 ± 1.0	2.7 ± 0.9	<i>P</i> < 0.05 <sup>1,2</sup>
Relaxation (%)	94.7 ± 8.0	85.2 ± 12.7	87.1 ± 12	

<sup>1</sup>Between IBS and SLE; <sup>2</sup>Between IBS and SSc; <sup>3</sup>Between SLE and SSc. IBS: Irritable bowel syndrome; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis.

tients with SLE and SSc (Figure 1). It was speculated that esophageal symptoms are mainly caused due to esophageal reflux<sup>[30,31]</sup>. However, in our study only patients with normal EGD were included and retrosternal burning was treated with PPI prior entering our study.

The most significant finding in our manometric study was that IBS patients showed very high peristaltic amplitudes in the proximal esophageal part and reduced

resting pressure of the lower esophagus sphincter (Figure 3) which was statistically significant different to SSc and SLE patients.

The analysis of esophageal peristaltic activity in patients with IBS showed controversial results. Diffuse peristaltic dysfunction with amplitudes > 150 mmHg and duration > 7 s<sup>[10,32]</sup>, simultan peristaltic<sup>[11]</sup> or also normal findings<sup>[12,33,34]</sup> have been reported. Reduced resting pres-

sure of LES has already been confirmed by others studies<sup>[9,11,30]</sup>. A pathophysiologic explanation or underlying pathomechanism is not known to date, however a correlation between small bowel or colonic dysfunction has been suggested<sup>[33,35]</sup>.

Our study clearly shows that IBS patients have pathologic motility patterns which are comparable to organic disorders (like SLE or SSc). However, the distribution of changes is different in IBS patients compared to SLE and SSc. A hypermotility of the proximal esophagus > 60 mmHg was seen in almost 50% of the patients. Our data suggest that altered esophageal motility is a common feature in IBS. Taken into consideration the small invasiveness of this method, esophageal manometry may have a place in the diagnostic work up of patients with suspected IBS, especially in the presence of dysphagic symptoms.

SLE patients showed significantly higher incidence of pathological manometric measurements concerning the resting pressure of LES (> 30 mmHg), the amplitudes of distal and middle peristalsis (> 160 mmHg and > 100 mmHg respectively) and the resting pressure of UES (< 40 mmHg) in comparison to IBS group. These findings, with exception to the resting pressure of the UES, are in contrast with the manometric findings in SSc, where most of the patients showed reduced peristaltic activity in the lower two thirds of esophagus (Figures 2 and 3).

Hyperperistalsis has been described from Peppercorn *et al.*<sup>[36]</sup> reporting cases of SLE with manometric features similar to diffuse esophageal spasm. In our study, we noticed peristaltic motility similar to nutcracker esophagus with biphasic waves and amplitudes up to 241 mmHg. Hypoperistalsis or aperistalsis, as previously described<sup>[37,38]</sup>, even in a small percentage, were not observed. These findings are consistent with Gutierrez *et al.*<sup>[38]</sup>. They described that such abnormalities are more often in SSc and mixed connective tissue diseases than in SLE.

In conclusion, this is - to the best of our knowledge - the first comparative study concerning esophageal motility among functional and autoimmune disorders. Although IBS, SLE and SSc patients showed different motility patterns, the intensity and frequency of dysphagia were comparable.

The esophageal peristalsis in patients with IBS appears to be more affected in the proximal part, whereas in the autoimmune disorders in the middle and distal one. Thus, smooth muscle changes might be associated with autoimmune diseases whereas striated muscles might be more affected in patients with IBS as suggested previously<sup>[39,40]</sup>. However, the absence of direct correlation between dysphagia score and manometric parameters in patients with IBS implies that, apart from motor dysfunction, visceral hypersensitivity plays an additional role to the pathology in IBS. In deed, visceral hypersensitivity in IBS patients has been documented in older and recent studies<sup>[34,41-45]</sup> pointing various lines of evidence for its relevance in the pathophysiology of IBS. Future studies are needed to further verify our data and to evaluate whether

different motility patterns can be used to diagnose IBS related motility changes of the esophagus.

## COMMENTS

### Background

Irritable bowel syndrome (IBS) is a functional disorder of the gastrointestinal tract with a still unclear pathophysiology. Although patients with IBS complain predominantly about manifestations concerning the lower gastrointestinal tract, esophageal symptoms are not uncommon.

### Research frontiers

Esophageal dysmotility is frequent in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) and systemic sclerosis (SSc). However, there is no comparative data available concerning esophageal motility between IBS patients and those with autoimmune disorders.

### Innovations and breakthroughs

This is the first comparative study concerning esophageal motility among functional and autoimmune disorders. Patients with IBS appear to have similar grade of dysphagia in comparison to patients with autoimmune disorders such as SLE and SSc. This study shows that IBS patients have pathologic esophageal motility patterns which are comparable to organic disorders.

### Applications

Esophageal manometry might have a place in the diagnostic work up of patients with suspected IBS.

### Terminology

Irritable bowel syndrome: Functional disorder of the gastrointestinal tract characterized mainly by symptoms as diarrhoea, constipation and diffuse abdominal pain. Systemic lupus erythematosus and systemic sclerosis are autoimmune diseases with esophageal and multiple other visceral manifestations.

### Peer review

The authors compared the esophageal motility between patients with IBS and patients with autoimmune disorders, such as SLE and SSc, at a single university prospective study. The outcome was calculating correlation coefficient between amplitudes, velocity and duration of the peristaltic waves throughout esophagus and patients' dysphagia for all three groups. It revealed that IBS patients showed similar pathologic dysphagia scores but were characterized from different motility patterns when compared to patients with autoimmune diseases. The results are interesting and suggest that esophageal manometry may have a place in the diagnostic work up of patients with suspected IBS, especially in the presence of dysphagic symptoms.

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## Diabetic neuropathy: An evaluation of the use of quercetin in the cecum of rats

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### Abstract

**AIM:** To investigate the effect of quercetin supplementation on the myenteric neurons and glia in the cecum of diabetic rats.

**METHODS:** Total preparations of the muscular tunic were prepared from the ceca of twenty-four rats divided into the following groups: control (C), control supplemented with quercetin (200 mg/kg quercetin body weight) (CQ), diabetic (D) and diabetic supplemented with quercetin (DQ). Immunohistochemical double staining technique was performed with HuC/D (general population)/nitric oxide synthase (nNOS), HuC-D/S-100 and VIP. Density analysis of the general neuronal population HuC/D-IR, the nNOS-IR (nitrergic subpopulation) and the enteric glial cells (S-100) was performed, and

the morphometry and the reduction in varicosity population (VIP-IR) in these populations were analyzed.

**RESULTS:** Diabetes promoted a significant reduction (25%) in the neuronal density of the HuC/D-IR (general population) and the nNOS-IR (nitrergic subpopulation) compared with the C group. Diabetes also significantly increased the areas of neurons, glial cells and VIP-IR varicosities. Supplementation with quercetin in the DQ group prevented neuronal loss in the general population and increased its area ( $P < 0.001$ ) and the area of nitrergic subpopulation ( $P < 0.001$ ), when compared to C group. Quercetin induced a VIP-IR and glial cells areas ( $P < 0.001$ ) in DQ group when compared to C, CQ and D groups.

**CONCLUSION:** In diabetes, quercetin exhibited a neuroprotective effect by maintaining the density of the general neuronal population but did not affect the density of the nNOS subpopulation.

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**Key words:** Diabetes; Myenteric plexus; Neuroprotection; Neuronal nitric oxide synthase; Vasoactive intestinal polypeptide; Enteric glia

**Core tip:** The present study is the first to report a neuroprotective effect of the flavonoid quercetin in the general population of enteric neurons in the cecum of rats with experimental diabetes mellitus. Quercetin did not reduce the loss of nitrergic neurons in the diabetic rats. This observation suggests that selective changes in the neurochemical code of enteric neurons occur in the presence of quercetin. We propose a causal link between the area and number of glial cells and the size of VIP-IR (reduction in varicosity population) varicosities. Although this link is not fully understood, these observations provide a basis for further studies to clarify the link between glia and VIP-IR varicosities.

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## INTRODUCTION

Diabetes affects the gastrointestinal tract causing changes in its motility, such as diarrhea, constipation and gastroparesis<sup>[1]</sup>. These symptoms are related to damage to the Enteric Nervous System (ENS) caused by diabetic neuropathy. Diabetes affects subpopulations of enteric neurons differently<sup>[2,3]</sup>, causing changes in neuronal size and density<sup>[2-6]</sup> and altering neurochemical code and neurotransmitters release<sup>[7]</sup>. The etiology of diabetic neuropathy is complex; hyperglycemia, which through various metabolic pathways including mitochondrial dysfunction and osmotic stress, can induce toxicity in neurons<sup>[8]</sup>; and oxidative stress, which results in a decrease in antioxidant capacity<sup>[9]</sup> through glucose oxidation, protein glycosylation and a decrease in the formation of reduced glutathione<sup>[10]</sup>, are among the causative factors. Reductions in the levels of non-enzymatic antioxidants such as ascorbic acid<sup>[11]</sup> and vitamin E enhance oxidative stress<sup>[12]</sup>.

Antioxidants are promising therapies for preventing and alleviating the debilitating clinical symptoms of diabetic neuropathy. Flavonoids, a family of polyphenolic compounds with a high antioxidant capacity<sup>[13]</sup>, might effectively protect against the pathology of diabetes. Quercetin is a flavonoid that is naturally present in various foods, such as onions, apples, broccoli, tea and red wine<sup>[14,15]</sup>. Quercetin has shown several beneficial pharmacological properties, such as antiperoxidative, anticarcinogenic, anti-inflammatory and antioxidant activities<sup>[16]</sup>. Ganglion neurons and their bundles of nerve fibers in the ENS are surrounded by numerous glial cells<sup>[17]</sup>. These glial cells play an important role in gastrointestinal physiology and pathophysiology, contributing to intestinal homeostasis, serving as a link between the nervous and immune systems<sup>[18]</sup> and influencing neurochemical phenotype<sup>[19]</sup>. The number of glial cells and neurons in the ENS are reduced by diabetes<sup>[2]</sup>.

The use of a potent antioxidant, such as quercetin, could mitigate this damage and alleviate the clinical symptoms of the disease. Because diabetes affects enteric neurons subpopulations differently<sup>[2,3]</sup>, the aim of this study was to investigate the potential for quercetin to mitigate of neuropathy. This aim was achieved by comparing the areas of the varicosities of vasoactive intestinal polypeptide (VIP)-containing neurons and the distributions of neuronal nitric oxide synthase (nNOS) and HuC/D (general population) containing neurons and glial cells in the ceca of diabetic and control rats.

## MATERIALS AND METHODS

### Animals

All the procedures involving animals were conducted in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA) and were reviewed and approved by the Ethics Committee on Animal Experiments (CEEA) at the Universidade Estadual de Maringá (State University of Maringá). For the present study, twenty-four adult male Wistar rats (Central Animal Facility at the State University of Maringá) were used. At 88 d of age, weighing 360 g on average, the animals were transferred to the vivarium Sector of the Morphological Sciences Department, where they were housed in individual cages maintained under controlled environmental temperature conditions of  $(22 \pm 2 \text{ }^\circ\text{C})$  and light/dark cycle (12/12 h) with *ad libitum* access to a water fountain and food (Nuvilab<sup>®</sup>).

After a 2-d period of adaptation to the new environment, rats were weighed and the 120-d trial period monitoring began. At this time, rats were divided randomly into four groups, each containing 6 animals, that received the following treatments: control (C), control supplemented with quercetin (200 mg/kg quercetin body weight) (CQ), diabetic (D) and diabetic supplemented with quercetin (DQ).

To induce diabetes, rats from the D and DQ groups were fasted for fourteen hours and then received an intravenous injection (penile vein) of streptozotocin (STZ) (Sigma, St. Louis, MO) at a dose of 35 mg/kg body weight, dissolved in citrate buffer 10 mmol/L (pH 4.5). Four days after the induction, blood glucose was measured (Accu-Chek Active, Roche Diagnostics GmbH, Mannheim, BW, Germany) to confirm the establishment of experimental diabetes. All the animals in the D and DQ groups had glucose levels above 210 mg/dL.

Starting on the fourth day of the experiment, animals in the CQ and DQ groups were weighed weekly and their water intake was measured. These measurements were used to calculate the dilutions required to ensure each animal in the CQ and DQ groups received 200 mg/kg per day of quercetin in their drinking water. Animals in C and D groups received water without supplementation. After 120 d (210-d-old), the rats were euthanized following anesthesia with Thiopental<sup>®</sup> (40 mg/kg *ip*; Abbott Laboratories, Chicago, IL). Blood was collected by cardiac puncture and blood glucose concentration measured using the glucose oxidase method<sup>[20]</sup>.

### Cecum collection and processing

The ceca were removed, washed in phosphate buffered saline (PBS; 0.1 mol/L pH 7.4) and filled with and immersed in Zamboni fixative solution<sup>[21]</sup> for 18 h at 4 °C. Following fixation, ceca were opened along their mesenteric borders and washed with 80% alcohol until the excess fixative was removed. Then, dehydration was performed in 95% and 100% EtOH, followed by diaphanization in xylene and sequential rehydration in 100%, 90%, 80% and 50% EtOH and finally PBS. Individual

**Table 1 Primary and secondary antibodies used for immunohistochemistry**

Primary	Host	Dilution dose	Company	Secondary	Dilution dose	Company
HuC/D	Mouse	1:500	Molecular Probes, Invitrogen	Anti-mouse Alexa Fluor 488	1:500	Molecular probes, Invitrogen
nNOS	Rabbit	1:500	Zymed	Anti-rabbit Alexa Fluor 546	1:500	Molecular probes, Invitrogen
S-100	Rabbit	1:500	Molecular Probes, Invitrogen	Anti-rabbit Alexa Fluor 546	1:500	Molecular probes, Invitrogen
VIP	Rabbit	1:500	Península Laboratories, Inc.	Anti-rabbit Alexa Fluor 546	1:500	Molecular probes, Invitrogen

HuC/D: General population; nNOS: Neuronal nitric oxide synthase; VIP: Vasoactive intestinal polypeptide.

**Table 2 Circumference area of the cecum and the correction factor used to calculate the neuronal density (mean  $\pm$  SEM)**

Groups	<i>n</i>	Area (cm <sup>2</sup> )	Correction factor
C	6	8.1 $\pm$ 1.0	Not applicable
CQ	6	7.5 $\pm$ 0.5	0.9
D	6	13.1 $\pm$ 0.7 <sup>b</sup>	1.6
DQ	6	10.9 $\pm$ 1.2	1.3

<sup>b</sup>*P* < 0.01 vs C group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin.

ceca were cut into small segments (approximately 2 cm<sup>2</sup>) that were subsequently microdissected under a stereomicroscope to remove the mucosa and submucosa and reveal the tunica muscularis.

### Immunohistochemistry

Three tissues sections per animal underwent immunohistochemical staining. One section was double-labeled to reveal immunoreactivity for HuC/D (general population) and nNOS. A second section was stained for HuC/D and S-100 (a glial protein), and a third section was stained to reveal immunoreactivity for vasoactive intestinal peptide (VIP). Tissues were washed twice in PBS containing 0.5% Triton X-100 for 10 min followed by one hour incubation in a blocking solution of PBS containing 2% BSA and 10% goat serum at room temperature under constant agitation. Tissues were then incubated for 48 h under agitation at room temperature in solutions of PBS containing primary antisera at the dilutions indicated in Table 1, 2% BSA, 0.5% Triton X-100 and 2% goat serum. Tissues were washed in PBS containing 0.5% Triton X-100 and incubated for 2 h at room temperature in solutions of PBS containing the appropriate secondary antisera at the dilutions indicated in Table 1, 2% BSA, 0.5% Triton X-100 and 2% goat serum. Tissues were then washed in PBS containing 0.5% Triton X-100 three times for 10 min and mounted on slides with 10% PBS in glycerol.

### Quantitative analysis of immunoreactive myenteric neurons

Analysis was performed by sampling the antimesenteric

basal region<sup>[22]</sup>. High-resolution micrographs of stained tissue were captured using an AxioCam MRC camera (Carl Zeiss, Jena, Germany) coupled to an Axioshop Plus fluorescence microscope (Carl Zeiss, Jena, Germany) with Axio Vision software (v. 4.6). Images were subsequently analyzed using Image-Pro Plus (v. 4.5.029; Media Cybernetics, Silver Spring, MD) to quantify the neurons and glia. For each animal, all the neurons and glial cells present in 30 images captured at  $\times 20$  magnification were manually identified and counted. The area of each image was approximately 0.2041 mm<sup>2</sup> and the total quantified area was 6.123 mm<sup>2</sup>. The results were expressed per square centimetre.

### Neuronal density correction

According to Cowen *et al.*<sup>[23]</sup>, pathological processes can change organ size, which can scatter the neurons. Therefore, the results of the neuronal and glial quantification were corrected for the changes in cecum size caused by diabetes (Table 2). For this correction, the cecum of each animal was outlined on cardboard and the images were transferred to the Image-Pro Plus software to measure the perimeter of each animal's cecum. The average area in cm<sup>2</sup> of the cecum in each group was used to calculate the correction factor and the factor was then applied to the quantitative results for each animal in the CQ, DQ and D groups (Tables 2 and 3).

### Morphometric analysis of immunoreactive myenteric neurons

Images of ganglia were captured using a 20  $\times$  objective for HuC/D-, nNOS- and S-100-immunoreactivity and a 40  $\times$  objective for VIP-immunoreactivity. Morphometric analyses were performed using the image analysis software Image Pro-Plus. The areas of 100 neuronal cell bodies (HuC/D-IR, nNOS-IR) and glial cells (S-100-IR) were measured per animal. For VIP, the areas of 400 varicosities per animal were measured. Varicosity measurements were performed using a digital zoom of  $\times 800$ , maintaining the original calibration of the captured image.

### Statistical analysis

Statistical analysis of the quantitative data was performed

**Table 3** Neuronal and glial density in the myenteric plexus of the cecum (mean  $\pm$  SEM)

Groups	HuC/D	nNOS	S-100	Ratio HuC-D/nNOS	Ratio HuC-D/S-100
C	5492 $\pm$ 81	2028 $\pm$ 102	6343 $\pm$ 367	2.7 $\pm$ 0.09	1.2 $\pm$ 0.05
CQ	5104 $\pm$ 132	1826 $\pm$ 124	8615 $\pm$ 318 <sup>b</sup>	2.9 $\pm$ 0.20	1.7 $\pm$ 0.07 <sup>a,c</sup>
D	4121 $\pm$ 325 <sup>b</sup>	1511 $\pm$ 162 <sup>a</sup>	5708 $\pm$ 322	2.8 $\pm$ 0.18	1.4 $\pm$ 0.16
DQ	5060 $\pm$ 25 <sup>c</sup>	1500 $\pm$ 59 <sup>a</sup>	5998 $\pm$ 269	3.4 $\pm$ 0.16 <sup>a</sup>	1.2 $\pm$ 0.04 <sup>c</sup>

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs C group; <sup>c</sup> $P < 0.05$  vs D group; <sup>e</sup> $P < 0.05$  vs DQ group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin.

using Statistica 7.1 and GraphPad Prism 3.1, with data expressed as the means  $\pm$  SEM. Morphometric data were set in delineation blocks and analyzed by Tukey's test. For the other results, we performed one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was 5%.

## RESULTS

Diabetes was induced by STZ administration in the D and DQ groups, as demonstrated by the assessment of blood glucose (Table 4). Hyperglycemia was accompanied by a significant reduction in the body weights in the D and DQ groups when compared with the control (C) group. Other symptoms typical of diabetes, such as polydipsia (increased water intake) and polyuria (increased urine output), were also observed (Table 4). In addition, the D group demonstrated a significant dilatation (61%) of the cecum compared with the C group ( $P < 0.01$ ). Treatment with quercetin helped to reduce the diabetes-associated dilation of the cecum in DQ group to 34% of the controls (Table 2), which was not significantly different from the C group ( $P > 0.05$ ). However, quercetin treatment did not affect the hyperglycemia, polydipsia or polyurea induced by STZ (Table 4).

### Morphology of HuC/D, nNOS, S-100 and VIP immunoreactivity in the cecum myenteric plexus

HuC/D-IR and nNOS-IR neuronal cell bodies were observed within the ganglia and along the interganglionic nerve tracks. The intensity of the immunofluorescence in neurons was heterogeneous within the four groups studied (Figure 1). We observed that the nitrergic population was usually located peripherally in the ganglion. Glial cells were present in all ganglia and along the nerve tracks. However, more glial cells were found in the nerve tracks of the CQ group than the other groups (Figure 2E and F). The VIP-IR varicosities were distributed throughout the tunica muscularis. However, in the D and DQ groups a reduction in the number of nerve fibers was observed (Figure 3C and D).

### Neuronal and glial density

After correction for cecum dilatation (Table 2), we ob-

**Table 4** Animal parameters (mean  $\pm$  SEM)

Groups	Starting weight (g)	Final weight (g)	Blood glucose (mg/dL)	Water intake (mL/d)	Feed intake (g/d)	Urine volume (mL/d)
C	341 $\pm$ 9	517 $\pm$ 13	138 $\pm$ 5	42 $\pm$ 3	37 $\pm$ 3	11 $\pm$ 2
CQ	366 $\pm$ 14	510 $\pm$ 10	146 $\pm$ 8	45 $\pm$ 3	34 $\pm$ 5	12 $\pm$ 2
D	333 $\pm$ 4	301 $\pm$ 21 <sup>a</sup>	518 $\pm$ 19 <sup>a</sup>	113 $\pm$ 16 <sup>a</sup>	44 $\pm$ 6	66 $\pm$ 10 <sup>a</sup>
DQ	368 $\pm$ 6	292 $\pm$ 8 <sup>a</sup>	532 $\pm$ 35 <sup>a</sup>	103 $\pm$ 22 <sup>a</sup>	30 $\pm$ 7	53 $\pm$ 13 <sup>a</sup>

<sup>a</sup> $P < 0.05$  vs C group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin.

served a reduction in the general population (HuC/D) of myenteric neurons in the ceca of diabetic rats compared with the C group ( $P < 0.001$ ). The CQ and DQ groups were not significantly different from the C group ( $P > 0.05$ ) (Table 3). There was a significant reduction in nNOS-IR neurons in D and DQ groups compared with the C group ( $P < 0.05$ ). Quercetin supplementation did not alter the cell densities between the D and DQ groups in this subpopulation ( $P > 0.05$ ). Double labeling showed that the ratio of HuC-D/nNOS was similar in the C, D and CQ groups but was significantly reduced in the DQ group (Table 3). Quantitative analysis of glia (S-100-IR) showed a significant increase in the CQ group compared with the C group ( $P < 0.001$ ). The D and DQ groups were not different from the C group ( $P > 0.05$ ). The ratio of glial cells to neurons (HuC-D/S-100) were similar in groups C, D and DQ, but were significantly increased in the CQ group when compared with the C and DQ groups ( $P < 0.05$ ) (Table 3).

### Morphometric analysis

**Neuronal population HuC/D-IR and nNOS-IR and glial cells:** The average HuC/D-IR and nNOS-IR neuronal areas in the C and CQ groups were not significantly different ( $P > 0.05$ ). However, there was an increase in the neuronal area in the D group when compared with the C group ( $P < 0.05$ ) and an even larger increase in the average area of the DQ group when compared with the D group ( $P < 0.05$ ). The mean areas of the HuC/D-IR and nNOS-IR neuron cell bodies are shown in Table 5. We observed a significant increase in the average area of the glia in the D group compared with the C, CQ and DQ groups, and a significant decrease in the DQ group compared with the C, CQ and D groups (Table 5).

**VIP-IR varicosities:** Fluorescence intensity was lower in VIP-IR varicosities in the DQ group compared with the other groups (Figure 3). We found significant differences in the diameter of the varicosities among the four groups studied; the CQ group had an increase in the average area of varicosities compared with the C group ( $P < 0.01$ ), a larger increase was observed in the D group compared with the C and CQ groups ( $P < 0.01$ ). In contrast, a decrease in the area of varicosities was found in the DQ group compared with all the other groups ( $P < 0.001$ ) (Table 5).

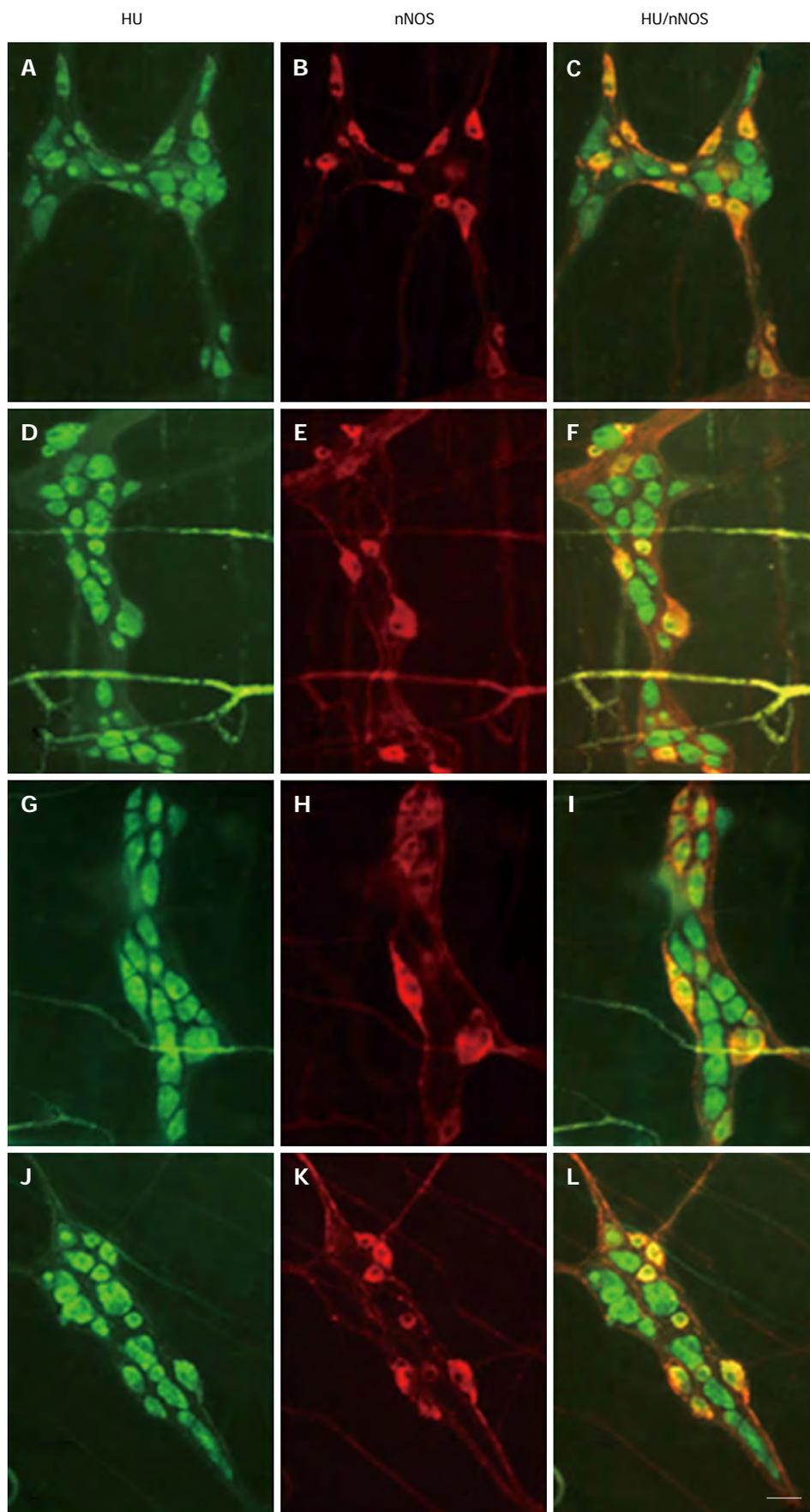


Figure 1 Representative micrographs showing immunoreactivity to general (green) and nitrergic (red) in the myenteric plexus of the rat cecum: A-C: Control group; D-F: Quercetin supplemented control group; G-I: Diabetic group; J-L: Quercetin supplemented diabetic group. Scale bar = 50  $\mu$ m. nNOS: Neuronal nitric oxide synthase.

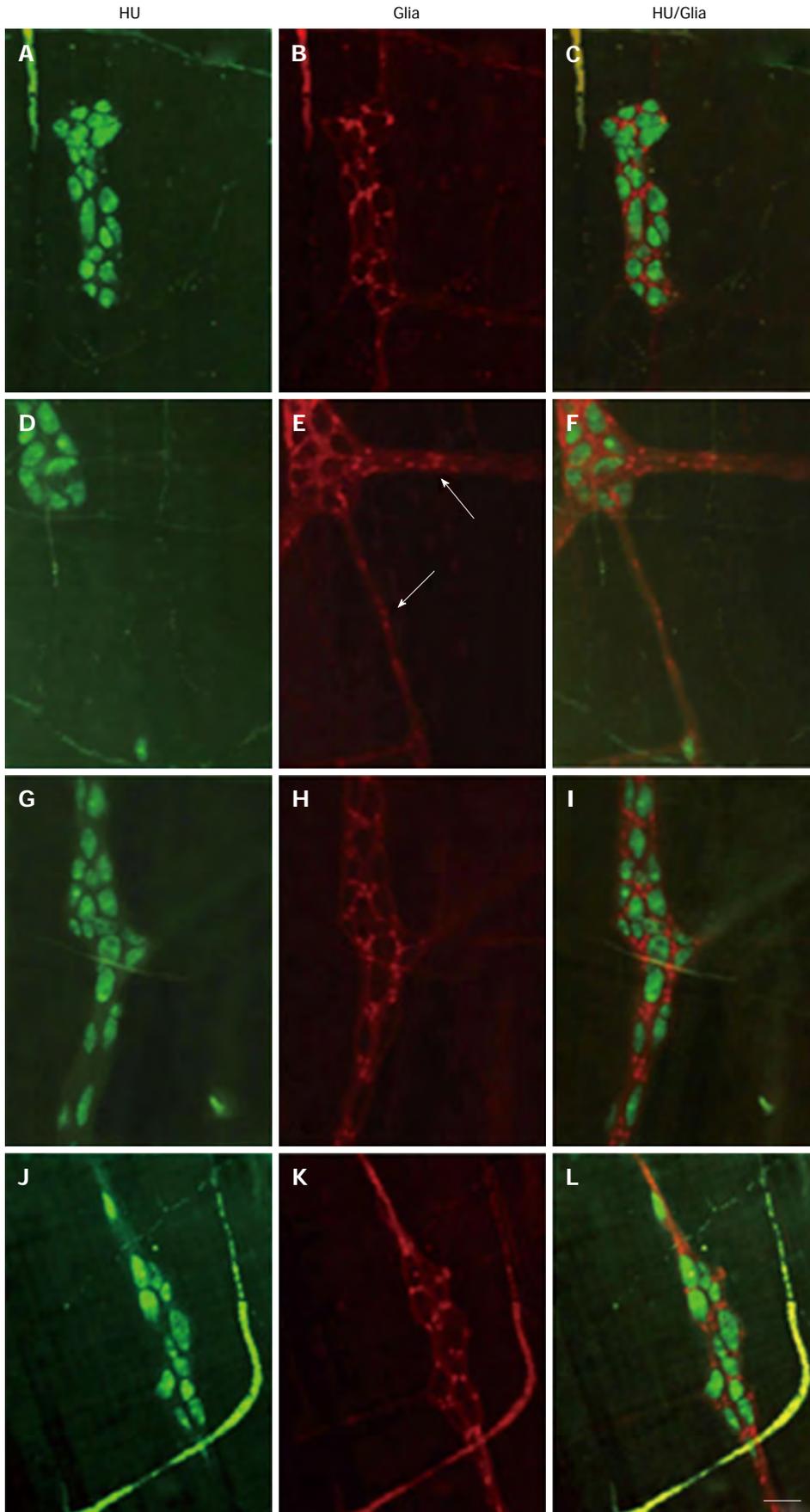


Figure 2 Representative micrographs showing immunoreactivity to HuC/D (green) and S-100 (red) in the myenteric plexus of the rat cecum. A-C: Control group; D-F: Quercetin supplemented control group; G-I, Diabetic group; J-L: Quercetin supplemented diabetic group. White arrows indicate glial cells present in nerve fibers. Scale bar = 50  $\mu$ m.

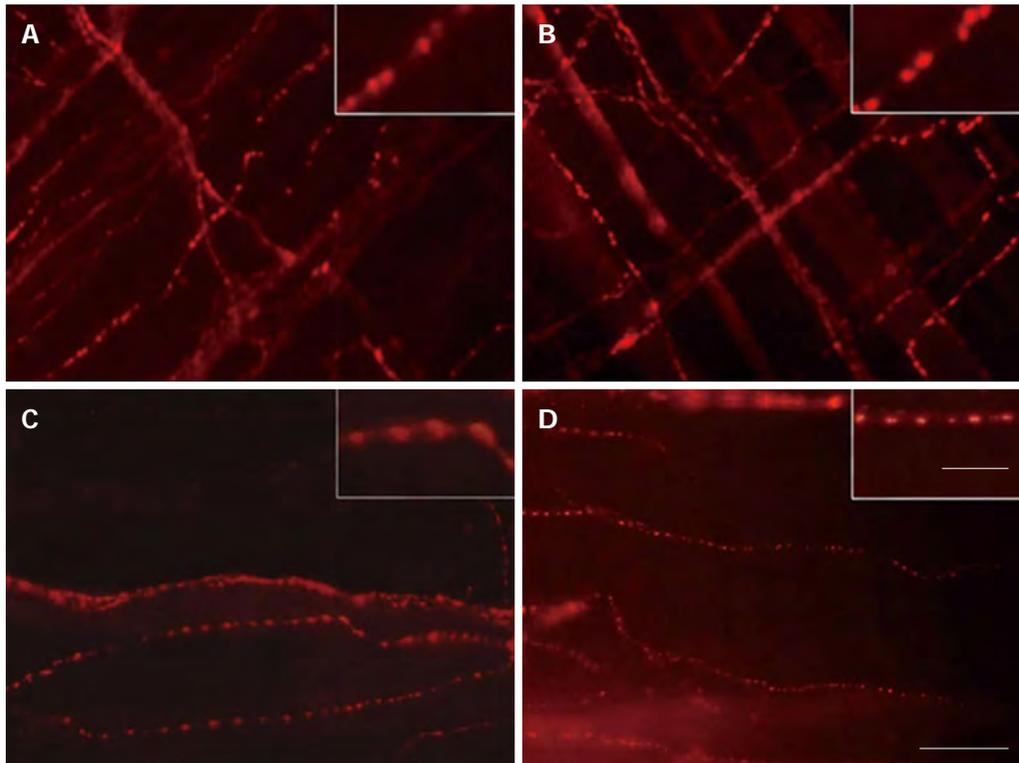


Figure 3 Representative micrographs showing immunoreactivity to vasoactive intestinal peptide in the myenteric plexus of the rat cecum. A: Control group; B: Quercetin supplemented control group; C: Diabetic group; D: Quercetin supplemented diabetic group. Magnified inserts show varicosities of the individual nerve fibers. Note the enlarged appearance of the varicosities in the diabetic group (C) and the reduced varicosities in the quercetin diabetic group (D) compared with the controls (A and B). Scale bars = 50  $\mu\text{m}$  for main panels, 15  $\mu\text{m}$  for inserts.

**Table 5 Mean cell body area of the neurons, glia and vasoactive intestinal polypeptide-IR varicosities (mean  $\pm$  SEM)**

Groups	HuC/D	nNOS	S-100	VIP
C	505 $\pm$ 11	455 $\pm$ 10	35.1 $\pm$ 0.5	4.63 $\pm$ 0.03
CQ	501 $\pm$ 12	430 $\pm$ 11	34.8 $\pm$ 0.4	4.88 $\pm$ 0.03 <sup>b</sup>
D	561 $\pm$ 13 <sup>a</sup>	539 $\pm$ 12 <sup>a</sup>	36.9 $\pm$ 0.5 <sup>a</sup>	5.12 $\pm$ 0.04 <sup>b</sup>
DQ	644 $\pm$ 16 <sup>b,c</sup>	606 $\pm$ 15 <sup>b,c</sup>	29.6 $\pm$ 0.4 <sup>b</sup>	3.97 $\pm$ 0.03 <sup>b</sup>

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 *vs* C group; <sup>c</sup>*P* < 0.05 *vs* D group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin; HuC/D: General population; nNOS: Neuronal nitric oxide synthase; VIP: Vasoactive intestinal polypeptide.

## DISCUSSION

STZ administration to animals in the D and DQ groups promoted typical characteristics of diabetes mellitus, including hyperglycemia, polyuria, polydipsia and weight loss. Quercetin treatment did not affect these measurements in control or diabetic rats suggesting that supplementation with this antioxidant did not influence metabolic pathways linked to weight gain or the mobilization of energy substrates in these animals.

Rats in the D group exhibited 61% cecum dilation compared with animals in the C group, so we used a correction factor to quantify neuronal density. The correction factor was required to ensure the results presented reflected real changes in the neuronal/glial population

density rather than the dispersion of these populations as a consequence of the dilation. Previous studies have shown that diabetes promotes dilation of the small intestine<sup>[6,24]</sup> and the large intestine<sup>[4,25,26]</sup>.

A reduction (25%) in the density of myenteric neurons that were immunoreactive to HuC/D in the D group compared with the C group was observed. Earlier studies in our laboratory showed neuronal loss in different gastrointestinal segments, including the stomach<sup>[27]</sup>, duodenum<sup>[28]</sup>, ileum<sup>[6]</sup>, proximal colon<sup>[29]</sup> and cecum<sup>[4,26]</sup>. These alterations may be attributed to the reduction in antioxidant defenses and the concomitant intensification of oxidative stress in the cells<sup>[10]</sup>. Free radicals can react with DNA, proteins and lipids and these reactions could cause nerve damage<sup>[30]</sup>, which results in the gastrointestinal motility disorders that are typical of diabetes<sup>[31]</sup>.

In the DQ group, quercetin promoted a preservation of neuronal density (HuC/D-IR) of 18% compared with the D group. The density in the DQ group was similar that observed in the C group. This preservation may be attributed to the antioxidant potential of quercetin<sup>[16]</sup>, which would minimize oxidative stress, preventing cell death by necrosis or apoptosis<sup>[32]</sup>. The ability of quercetin to reduce superoxide anions ( $\text{O}_2^{\cdot-}$ ), singlet oxygen and hydroxyl radicals ( $\text{HO}^{\cdot}$ ), could also prevent lipid peroxidation caused by these molecules<sup>[33]</sup>. Finally, quercetin may induce the gene expression of antioxidant enzymes, increasing glutathione levels (GSH) and conferring neuroprotection<sup>[34]</sup>. In the present study, we

found a significant reduction in the density of nitrergic neurons in the cecum of the rats from the D group (25%) and the DQ group (26%) compared with the C group. These decreases in nitrergic density may be attributed to the duration of the diabetes (17 wk). Previous studies have shown an accumulation of advanced glycation end-products, which result in oxidative stress and in neuronal apoptosis, begins in the twelfth week of diabetes<sup>[35,36]</sup>. Reductions in the number of neurons and/or nNOS activity were also observed in the stomach of diabetic rats<sup>[37,38]</sup>. However, depending on the model, the duration of diabetes and the techniques used to assess these changes, we can find an increase<sup>[39]</sup>, a decrease<sup>[35,37]</sup>, or no change<sup>[6]</sup> in the number of nitrergic neurons and/or nNOS levels and activity in different regions of the gastrointestinal tract. According to Shotton and colleagues<sup>[3]</sup>, these inconsistencies may be explained by regional or neuronal subpopulation differences and/or by the existence of multiple stages in the development of neuropathy. In the current study, quercetin treatment did not prevent the reduction of nitrergic neurons compared with D group as it did for the general neuronal population (HuC/D-IR). These findings are of great interest, as quercetin seems not only to prevent neuronal loss but also to direct the chemical coding of the neurons it protects.

In the CQ group, we observed an increase in the VIP-IR varicosity areas compared with the C group ( $P < 0.01$ ), similar data were obtained by Alves *et al.*<sup>[40]</sup> who studied the effect of supplementation with L-glutamine in the jejunum of normoglycemic and diabetic rats. VIP is an inhibitory neuropeptide that has an important role in regulating glial cell proliferation, modulating cell plasticity, stimulating the release of neuroprotective factors and secreting gliotransmitters/gliopeptides that are involved in intercellular communication<sup>[41]</sup>. In the present study, there was a significant increase (36%) in the number of glial cells in the CQ group compared with the C group ( $P < 0.001$ ). In the CNS, VIP promotes astrocytic proliferation<sup>[41,42]</sup>; a quercetin-induced increase in VIP may cause an increase in enteric glia in the same way. Furthermore, VIP is capable of stimulating the production of neurotrophic factors by glia in the CNS<sup>[43]</sup>. An interesting finding in the present study was that there was a greater increase in the number of glia within the fiber tracts rather than within the ganglia in the CQ group. Fiber tracts also contain numerous VIP-IR varicosities.

However, the observation that diabetic rats (group D) demonstrated an increase in the area of VIP-IR varicosities compared with the C group ( $P < 0.01$ ), without a concomitant increase in the density of glial cells argues against the hypothesis of a direct causal relationship between VIP and glia. These seemingly conflicting observations may be explained by other factors that might be present in the diabetic state. There are published reports of increased expression and release of interleukin (IL)-1 beta in STZ-induced diabetic rats<sup>[44]</sup> and in human monocytes treated *in vitro* with different concentrations of glu-

cose<sup>[45]</sup>. Studies by Rühl and colleagues<sup>[46]</sup> demonstrated a combined response of IL-1 beta and IL-10 that lead to a reduction in glial cell proliferation. Thus, despite an increase in IL-10 resulting from increased VIP expression the diabetic state could also be promoting an increase of IL-1 beta and together these two cytokines would inhibit glial cell proliferation in the D group. Regardless of the relationship between VIP and glia, which requires further study, the increase in the size of the VIP-IR varicosities in the diabetic rats may be explained by an increased expression of VIP as a compensatory effect due to neuronal loss<sup>[47]</sup>, or it may be a reflection of neuronal plasticity to maintain the survival of the neurons in response to the pathophysiological conditions of diabetic neuropathy<sup>[48]</sup>. This conclusion could be supported by studies demonstrating an important role for VIP in neuroprotection<sup>[49,50]</sup>, perhaps by scavenging reactive oxygen species as demonstrated *in vitro*<sup>[49]</sup> and *in vivo*<sup>[51]</sup>.

Morphometric analysis of the general neuronal population (HuC/D-IR) and the nitrergic (nNOS-IR) subpopulation showed a significant increase in the neuronal area in the D group compared with the C group. An increase in neuronal area is a frequent finding in diabetic animals<sup>[4,6]</sup>. Hypertrophy was also observed in subpopulations of enteric neurons in the diabetic rats, including the VIP-IR neurons<sup>[40,47]</sup>, nNOS-IR<sup>[5]</sup> and NADH diaphorase-positive neurons<sup>[52]</sup>. In the present study, we observed a significant increase in the neuronal area of the general (HuC/D) and nitrergic (nNOS-IR) populations in the DQ group when compared with the other studied groups (Table 5). This event could be explained a the reduction in glial function and metabolism, which was suggested by the decrease in glial cell area and which could reduce the production of neurotrophic factors leading to a loss of control over the processes of synthesis, potentially changing the neurochemical phenotype of the neurons. Evidence that enteric glial cells can produce neurotrophic factors, such as nerve growth factor, brain derived neurotrophic factor and neurotrophin 3, that modulate neuronal gene expression and possibly the enteric neurophenotype has been observed<sup>[53]</sup>. Additionally, neurotrophic factors play a critical role in regulating the synthesis of neurotransmitters and neuropeptides and in influencing neuronal morphology<sup>[54]</sup>.

In summary, we concluded that in diabetic rats, quercetin exhibited a neuroprotective effect due to its antioxidant action. This action is independent of diabetes-induced changes in hyperglycemia, polydipsia, polyurea and weight loss. Interestingly, while quercetin was able to reduce the loss of myenteric neurons, it did not reduce the loss of nitrergic neurons suggesting a selective change in the neurochemical coding of the enteric neurons during quercetin treatment. Quercetin treatment increased the area of VIP-IR varicosities and concurrently increased the density of enteric glia in control animals. In diabetic rats, there is a disconnection between these observations: quercetin does not increase glial density, but does decrease VIP-IR varicosity area. While there are

data in the literature to support a causal link between VIP and glia and to suggest a connection to neuronal loss and changes in chemical coding, the molecular mechanisms and the relationships between these observations remain to be elucidated.

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## COMMENTS

### Background

Hyperglycemia from diabetes mellitus (DM) may cause long-term neuropathic abnormalities that affect the autonomic nervous system. In the gastrointestinal tract, neurodegeneration and morphological changes in the neurons and glia cells of the enteric nervous system (ENS) are observed. These changes are related to the oxidative stress of diabetes. Hyperglycemia is responsible for an increase in oxidative stress and a decrease in antioxidant capacity. In this context, the use of flavonoids, a family of polyphenolic compounds with a high antioxidant capacity, might be effective in protecting against the pathology of diabetes. Quercetin is a flavonoid that is present in various foods, such as grapes, and their derivatives. Several studies have revealed the beneficial pharmacological effects of quercetin in biological systems, including its potent antioxidant effect. Thus, the use of quercetin is a promising therapy for the prevention of neurological disorders and can reduce the pathological conditions of diabetes.

### Research frontiers

Currently, diabetes is considered an epidemic that affects more than 300 million people worldwide. Its chronic nature combined with the severity of its complications and the necessary means to control them, makes diabetes a very costly disease for patients and for the healthcare system. Autonomic neuropathies, which are among the complications of diabetes, can trigger a wide range of gastrointestinal problems, such as nausea, vomiting, diarrhea, constipation and fecal incontinence, that cause discomfort and deeply affect the quality of life of patients with DM. In this context, studies evaluating therapeutic strategies that have the potential to improve or mitigate the degenerative damage to the enteric nervous system, such as the use of the flavonoid quercetin, may eventually contribute to an improved quality of life for these patients.

### Innovations and breakthroughs

Previous studies in their research group, using simple neuronal marking techniques, reported the absence of an effect of antioxidants on the cecum of rats with experimental DM. In this study, using immunohistochemical techniques, the authors could observe an effect of antioxidant treatment on the cecum in a rat DM model. In addition, using these techniques we observed this effect in both the general neuronal population and in specific neuronal subpopulations of the cecum ENS. Although, studies suggest a neuroprotective effect of quercetin in the central nervous system, there are only a few studies examining the effect of quercetin in the ENS of rats with experimental DM.

### Applications

The present study shows that quercetin could improve antioxidant capacity and thus protect the enteric nervous system in the cecum of streptozotocin-induced diabetic rats *in vivo*. When these effects are confirmed by further research, future application of quercetin as a therapeutic in the treatment of diabetic neuropathy may be merited. Another aspect of the present study is the identification of an apparent causal relationship between VIP and glia. This observation could provide a basis for the clarification of other research.

### Terminology

ENS is made of sensory neurons, interneurons and motoneurons and is divided into two major plexuses in the gastrointestinal tract: the myenteric and submucosal. VIP and the enzyme Nitric Oxide Synthase are neuronal sub-

populations of the ENS that express inhibitory neurotransmitters and are non-adrenergic and non-cholinergic. Enteric glia: a set of cells, which are similar to the astrocytes of the CNS, that are adhered to the ENS ganglion neurons and their nerve fiber bundles.

### Peer review

This paper concerns an interesting issue, however introduction is repetitive, and discussion is too long and should be reduced.

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## Full robot-assisted gastrectomy with intracorporeal robot-sewn anastomosis produces satisfying outcomes

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### Abstract

**AIM:** To evaluate the feasibility and safety of full robot-assisted gastrectomy with intracorporeal robot hand-sewn anastomosis in the treatment of gastric cancer.

**METHODS:** From September 2011 to March 2013, 110 consecutive patients with gastric cancer at the authors' institution were enrolled for robotic gastrectomies. According to tumor location, total gastrectomy, distal or proximal subtotal gastrectomy with D2 lymphadenectomy was fully performed by the da Vinci Robotic Surgical System. All construction, including Roux-en-Y jejunal limb, esophagojejunal, gastroduodenal and gastrojejunal anastomoses were fully carried out by the intracorporeal robot-sewn method. At the end of surgery, the specimen was removed through a 3-4 cm incision at the umbilicus trocar point. The details of the surgical technique are well illustrated. The benefits in terms of

surgical and oncologic outcomes are well documented, as well as the failure rate and postoperative complications.

**RESULTS:** From a total of 110 enrolled patients, radical gastrectomy could not be performed in 2 patients due to late stage disease; 1 patient was converted to laparotomy because of uncontrollable hemorrhage, and 1 obese patient was converted due to difficult exposure; 2 patients underwent extra-corporeal anastomosis by minilaparotomy to ensure adequate tumor margin. Robot-sewn anastomoses were successfully performed for 12 proximal, 38 distal and 54 total gastrectomies. The average surgical time was  $272.52 \pm 53.91$  min and the average amount of bleeding was  $80.78 \pm 32.37$  mL. The average number of harvested lymph nodes was  $23.1 \pm 5.3$ . All specimens showed adequate surgical margin. With regard to tumor staging, 26, 32 and 46 patients were staged as I, II and III, respectively. The average hospitalization time after surgery was 6.2 d. One patient experienced a duodenal stump anastomotic leak, which was mild and treated conservatively. One patient was readmitted for intra-abdominal infection and was treated conservatively. Jejunal afferent loop obstruction occurred in 1 patient, who underwent re-operation and recovered quickly.

**CONCLUSION:** This technique is feasible and can produce satisfying postoperative outcomes. It is also convenience and reliable for anastomoses in gastrectomy. Full robotic hand-sewn anastomosis may be a minimally invasive technique for gastrectomy surgery.

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**Key words:** Robotic surgery; Gastric cancer; Total gastrectomy; Esophagojejunal anastomosis

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## INTRODUCTION

Although laparoscopic gastrectomy has been widely performed worldwide, its role is still a matter of debate due to inherent difficulties in specific node dissection and intracorporeal anastomosis<sup>[1,2]</sup>. Recently, robotic surgery has been demonstrated to overcome the intrinsic limitations of a traditional laparoscopic approach, where the anatomical and operative conditions are similar to those encountered during gastric resection<sup>[3,4]</sup>. Several recent retrospective studies have reported that robotic surgery for the treatment of gastric cancer is feasible and can produce satisfying postoperative outcomes<sup>[5-7]</sup>. However, most studies have reported that anastomosis after robotic gastrectomy was carried out by extracorporeal hand-sewn sutures or an intracorporeal stapler.

Wristed instruments that allow seven degrees of freedom, tremor filtering, the ability to scale motions, and stereoscopic vision improve the surgeon's dexterity when fine manipulation of tissues in a close, fixed operating field or when hand-sewn sutures and knot tying are required<sup>[8]</sup>. In robotic surgery for other complex robotic procedures, such as urethral anastomosis in radical prostatectomy or valve replacement in cardiac surgery, several studies have reported that robot hand-sewn anastomosis was possible within a narrow space due to these distinct advantages<sup>[9-11]</sup>. Therefore, we believe that a robotic approach would also be relevant for laparoscopic D2 dissection and intracorporeal anastomosis by a full robot hand-sewn method.

To the best of our knowledge, no study has assessed the reliability of this hand-sewn technique or described its technical details, although it is a classic and feasible method. The current study aimed to assess the feasibility and safety of full robot-assisted total and subtotal gastrectomy with extended lymphadenectomy and intracorporeal robot-sewn anastomosis.

Here, we present the results of a preliminary study in which anastomosis after gastrectomy was successfully achieved by a robot-sewn technique. All procedures, including lymph node dissection and anastomosis, were completed by the robot, the so-called "full robot-assisted gastrectomy", which was different from previous robotic surgery for the treatment of gastric cancer.

## MATERIALS AND METHODS

All procedures were performed by the da Vinci Surgical System (Intuitive Surgical, Inc, Mountain View, CA, United States). We began using this system for gastric cancer surgery in May 2010 at Jinglin Hospital, affiliated to Nanjing University, China. From September 2011 to

March 2013, we conducted a prospective evaluation of the feasibility and safety of robot-assisted gastrectomy with intracorporeal robot hand-sewn anastomosis. During this time, all patients with histologically proven gastric cancer without organ invasion (T4) underwent preoperative work-up and examination. One hundred and ten consecutive patients diagnosed with gastric cancer were enrolled in this trial (details in Figure 1). Robotic anastomosis was performed by a surgeon (Dr. Jiang ZW) who had been involved with more than 100 cases of robotic-assisted gastrectomy before this trial. We obtained informed consent from all patients for administration of this robotic surgery anastomotic method.

Perioperative management was performed by adopting the measures of fast track surgery<sup>[12,13]</sup>. Preoperative short-time fasting and carbohydrate loading were introduced. Nasogastric decompression tubes were abandoned in all patients, unless absolutely necessary. When able, patients were given water from postoperative day 1, liquid diet was started on postoperative day 2, and soft diet was started on postoperative day 3. After 1 d of soft diet without complications, patients were discharged.

All data were collected prospectively. Operative time was calculated as the time between pneumoperitoneum induction and port-site closure. Intraoperative blood loss was measured by subtraction. Tumor staging and lymph node harvest rate were assessed by the pathology department. Surgical and oncologic outcomes were well documented. Patients were evaluated weekly with clinical examinations during the 30 d after discharge and then followed-up every 3 mo. We evaluated feasibility and safety of the procedure with the Clavien-Dindo classification, which categorizes surgical complications from grade 1 to 5 based on the invasiveness of the treatment required. Grade 1 requires no treatment; grade 2 requires medical therapy; grade 3a requires surgical, endoscopic, or radiologic intervention, but not general anesthesia; grade 3b requires general anesthesia; grade 4 represents life-threatening complications that require intensive care; and grade 5 represents death of the patient.

### *Patient and robot position, port placement*

The patient is moved to the 20° reverse Trendelenburg position under general anesthesia. The camera port (C) is inserted into the infra-umbilical area for a 12 mm trocar. After establishing 12-mmHg pneumoperitoneum, the other four ports are placed with the aid of camera visualization.

Two 8-mm Intuitive cannulae for robotic devices are placed under direct visualization 2-3 fingerbreadths below the costal margin at the right and left anterior axillary line, respectively (Figure 2, trocar A and E). The last 8-mm Intuitive cannula (B) is placed in the right paraumbilical area below the level of port A and at least one handbreadth away from the camera port.

One 12-mm trocar (D) is placed along the left mid-clavicular line, in the left paraumbilical area and at least

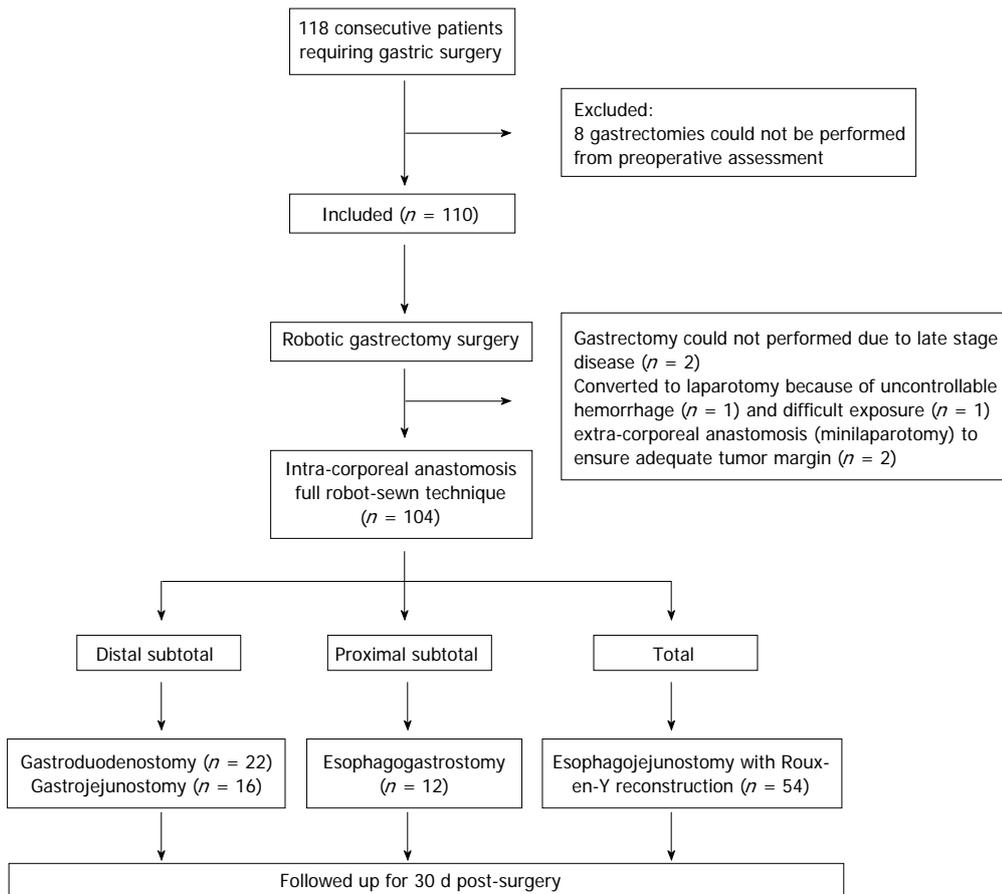


Figure 1 Flow diagram.

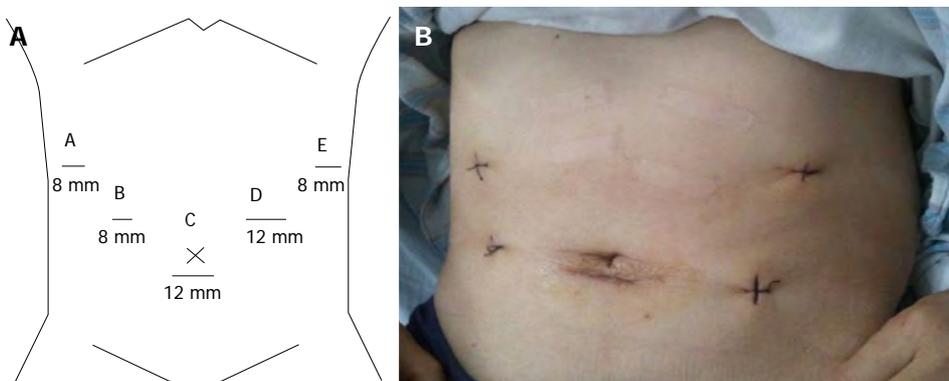


Figure 2 Placement of surgical ports. For A, B and E 8-mm ports were used. For C and D 12-mm ports were used. Port C was extended to 3 cm for specimen extraction from the abdominal cavity.

one handbreadth away from the camera port (C). The assistant who works on the patient's left side uses this port (D) to aid the surgeon during the robotic operation, such as insertion of an endo-stapler for resection of the duodenum, the stomach, or the abdominal esophagus and for placement of gauze or a suction device for clearing the operative field (Figure 2). After port placement, the robotic cart is installed from the patient's head.

In Japan and Europe, extended lymph node dissection (D2) is the standard of care for gastric cancer<sup>[14-16]</sup>.

Robotic gastrectomy with D2 lymph node dissection were performed according to the rules of the Japanese Research Society for Gastric Cancer<sup>[17,18]</sup>. Total gastrectomy, distal or proximal subtotal gastrectomy was decided according to tumor location. The lymphatic tissues are removed *en bloc* along the hepatic, splenic, left gastric artery and celiac trunk using an ultrasonic shear. The origins of these arteries are clearly identified and skeletonized, and the lymphatic tissue dissected away from the adventitia. The left gastric artery is then clipped or tied at its origin



Figure 3 Lymphatic tissues are removed *en bloc* along the hepatic, splenic, left gastric artery and celiac trunk using an ultrasonic shear. The origins of these arteries are clearly identified and skeletonized, and the lymphatic tissue dissected away from the adventitia. The left gastric artery is then clipped or tied at its origin.

(Figure 3). Once the lymphadenectomy is complete, the assistant divides the stomach, intestine or esophagus using multiple endostapler applications (Ethicon Endo-Surgery, Cincinnati, United States) from the 12 mm trocar D. The specimen, including the stomach, omentum, and the lymphatic tissue, are wrapped by an endobag. The specimen was extracted from the abdominal cavity through the intraumbilical port site extended to 3 cm.

#### **Distal subtotal gastrectomy with gastroduodenostomy (Billroth I) and gastrojejunostomy (Billroth II)**

After distal subtotal gastrectomy, 38 patients underwent gastroduodenostomy or gastrojejunostomy reconstruction. For gastroduodenostomy, the duodenum was resected by an endo-linear stapler inserted into the assistant's 12 mm trocar D. The remnant portion of the lesser curvature was resected by an endo-linear stapler, and the completely resected stomach was then wrapped by an endobag. The posterior walls of the duodenum and the stomach were approximated by continuous seromuscular sutures (Figure 4B and C); the duodenal stump was then opened by an ultrasonic shear. A continuous suture with interlocking of the full intestinal layers of the posterior and anterior wall of the duodenum and the stomach was then made (Figure 4D and F). Finally, the anterior wall of the anastomosis was reinforced by interrupted seromuscular sutures (Figure 4G). Sometimes, the duodenum was not transected until the posterior wall suturing of the gastroduodenostomy was finished. This method can facilitate pulling up the duodenum for anastomosis. The duodenum was totally dissected using an ultrasonic scalpel (Figure 4C and E). For gastrojejunostomy reconstruction, the jejunum which is about 20 cm away from the Treitz was brought up just below the remnant stomach for antecolic end-to-side anastomosis, which was achieved using the hand-sewn technique in the same manner.

#### **Proximal subtotal gastrectomy with esophagogastrostomy**

For esophagogastrostomy reconstruction after proxi-

mal subtotal gastrectomy, the remnant distal stomach in which the gastroepiploic arcade was preserved was brought up just below the dissociated esophagus for end-to-end anastomosis. Robotic interrupted suturing was performed to fix the distal gastric-remnant and esophagus together. A continuous suture with interlocking of the full layers of the posterior and anterior wall of the esophagus and the stomach was then made (Figure 5). Finally, the anterior wall of the anastomosis was reinforced by interrupted seromuscular sutures.

#### **Total gastrectomy with esophagojejunostomy and Roux-en-Y reconstruction**

Fifty-four esophagojejunostomies were performed using methods similar to those described above (Figure 6). After the total stomach was divided, the assistant aids the console surgeon in manipulating the bowel to identify the ligament of Treitz. The small bowel which is 15-20 cm away from the Treitz was brought up just below the dissociated esophagus for antecolic end-to-side anastomosis. Robotic needle holders are loaded with 3-0 absorbable sutures and interrupted suturing is performed to fix the jejunum and esophagus together. Then continuous interlocking suturing is performed between the posterior esophageal wall and seromuscular layer of the jejunum. A 2-3 cm incision is made in the jejunum to be anastomosed. The posterior wall of the esophagus is dissected for the half ring at about 1-2 cm above the cardia. The posterior esophageal and jejunal walls are sutured by a continuous interlocking suture (Figure 6).

Sometimes, especially when the tumor is small or adjacent to the cardia, the tumor cutting edge must be clearly identified. The esophagus is not transected until the posterior wall suturing of the esophagojejunostomy is finished (Figure 6K and L). This strategy can facilitate not only identification of the tumor cutting edge, but also pulling down the esophagus for anastomosis. The remaining half ring of the anterior wall of the esophagus is dissected using an ultrasonic scalpel.

After the anterior wall is clearly exposed, a continuous interlocking suture anterior of the anastomosis wall is performed. Finally, the anterior esophageal wall and anterior seromuscular layer of the jejunum are sutured using interrupted sutures (Figure 6D and E). The proximal jejunum 5 cm away from the esophagojejunal anastomotic stoma, is then transected by the assistant using a 45-mm cartridge endostapler (gold loads, Ethicon Endo-Surgery). The side-to-side jejunojunction and jejunal stump are achieved using the hand-sewn technique in the same manner (Figure 6F-J).

## **RESULTS**

Of the 110 patients enrolled in this trial, two patients could not undergo radical gastrectomy due to late stage disease; 1 patient was converted to laparotomy because of uncontrollable hemorrhage, and 1 obese patient was

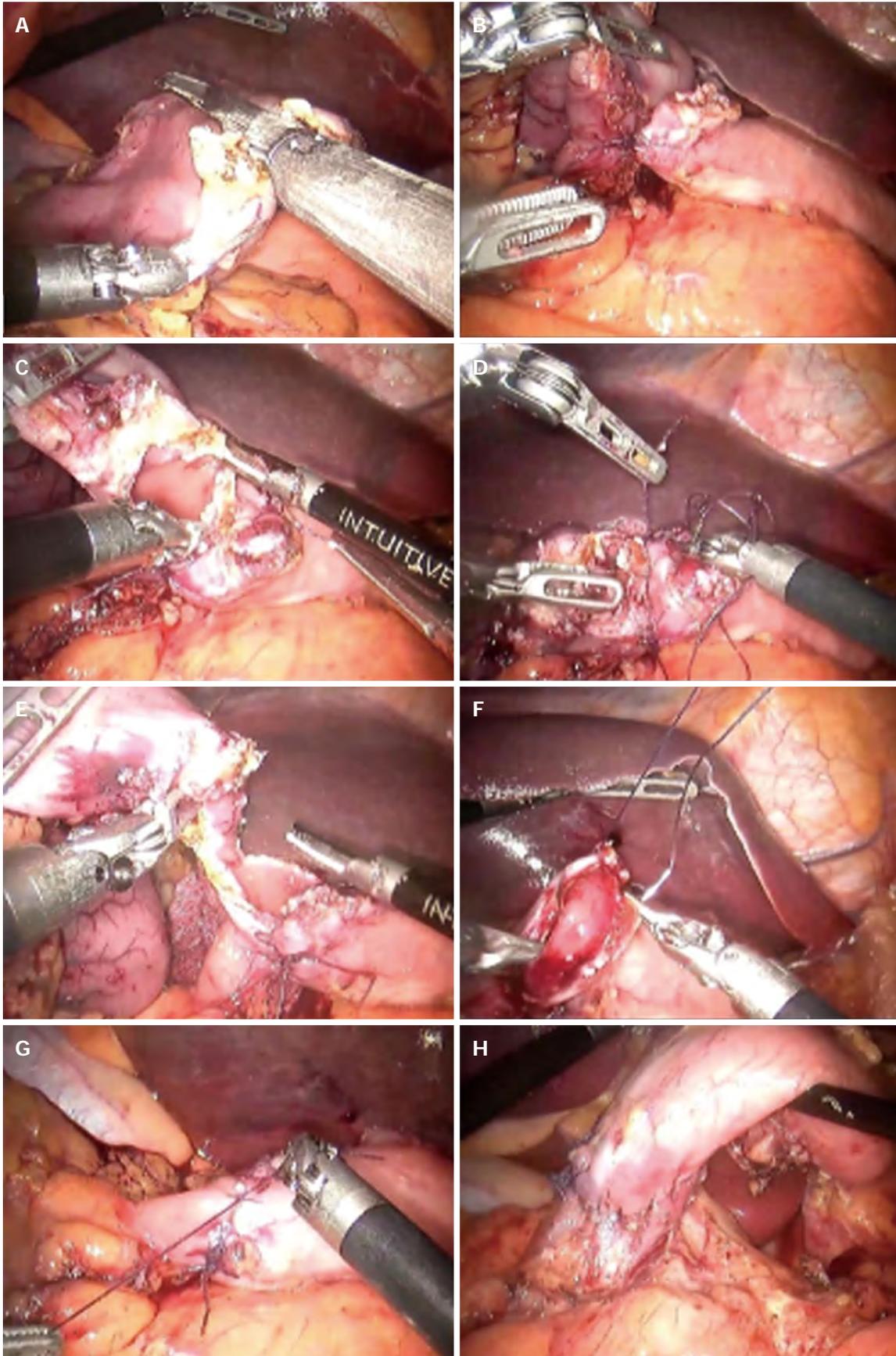
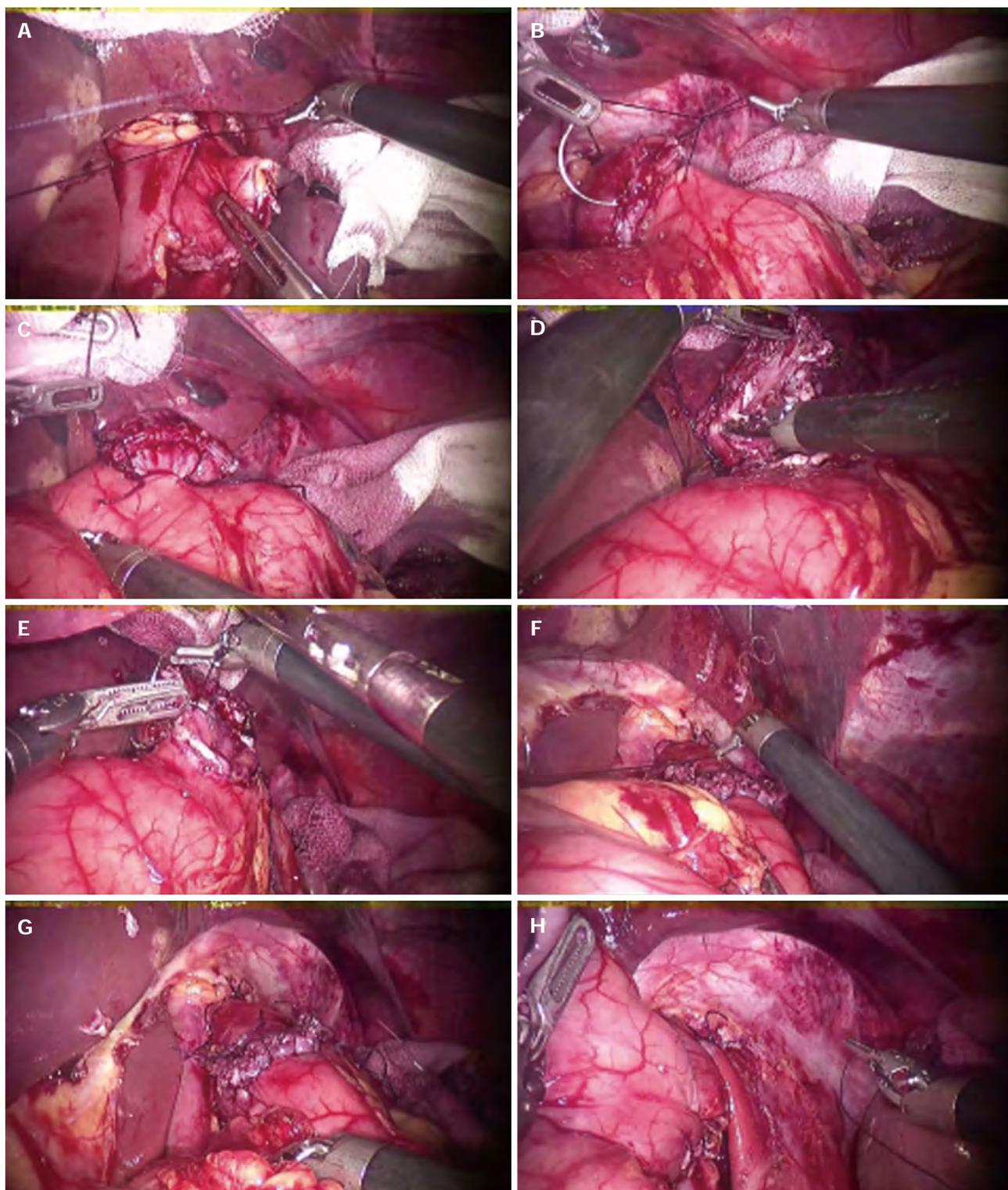


Figure 4 Distal subtotal gastrectomy with gastroduodenostomy (construction type of Billroth I ). A, B: Robotic anastomosis for gastroduodenostomy; C: Continuous seromuscular suture; D, E: Continuous interlocking suture for posterior wall; F: Continuous interlocking suture for anterior wall; G: Interrupted sero-muscular suture; H: Complete anastomosis.



**Figure 5** Proximal gastric resection with esophagogastrostomy. A: The terminal esophagus fully mobilized. Diaphragmatic crura are exposed and freed from the surrounding adipose and lymphatic tissue. The esophagus was stitched to the crura for better exposure; B-G: The remnant distal stomach was brought up just below the dissociated esophagus for end-to-end anastomosis; H: Complete anastomosis.

converted due to difficult exposure; 2 patients underwent extra-corporeal anastomosis by minilaparotomy to ensure adequate tumor margin. There were no cases of pancreatic or spleen injury during surgery.

Robot-assisted gastrectomy with total robot-sewn anastomosis were successfully performed in 104 cases,

including 66 males and 38 females with an average age of  $58.2 \pm 12.6$  years (range: 40-76 years) and body mass index (BMI) of  $22.12 \pm 4.64 \text{ kg/m}^2$  (range: 16-26  $\text{kg/m}^2$ ). Patient characteristics are presented in Table 1.

Fifty-four esophagojejunostomies with Roux-en-Y reconstruction for 54 total gastrectomies, 22 gastroduo-

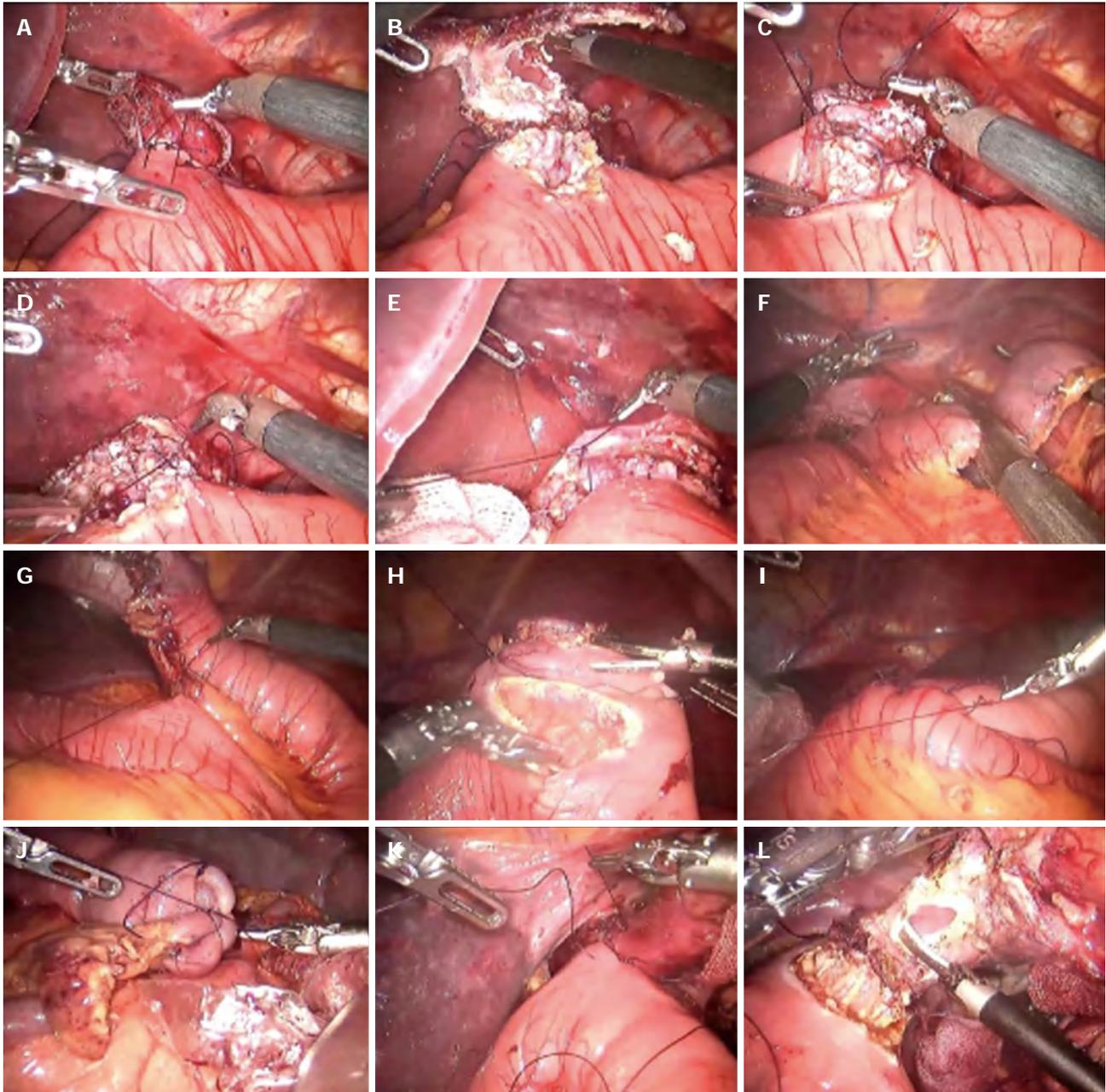


Figure 6 Total gastrectomy with esophagojejunostomy and Roux-en-Y reconstruction. A-E: The small bowel which is 15-20 cm away from the Treitz was brought up just below the dissociated esophagus for antecolic end-to-side anastomosis. Robotic anastomosis for esophagojejunostomy was performed in the same manner; F: The proximal jejunum 5 cm away from the esophagojejunal anastomotic stoma is transected by a 45-mm cartridge endostapler; G-I: The side-to-side jejunojunction and jejunal stump were achieved using the hand-sewn technique in the same manner; J: Jejunal stump was achieved using the hand-sewn technique in the same manner; K, L: Sometimes, the esophagus was not transected until the posterior wall suturing of the esophagojejunostomy was finished.

denostomies and 16 gastrojejunostomies for 38 distal subtotal gastrectomies, and 12 esophagogastromies for 12 proximal subtotal gastrectomies were successfully performed. The average operation time was  $272.52 \pm 53.91$  min, and median reconstruction time was  $45.8 \pm 26.0$  min (Table 2). The average amount of bleeding during surgery was approximately  $80.78 \pm 32.37$  mL. From the pathologic findings, the average number of harvested lymph nodes was  $23.1 \pm 5.3$ . No tumor specimens showed positive surgical margins. The final pathological staging was as follows: stage I, 26 cases; stage II, 32

cases; stage III, 46 cases.

The average time to first flatus and semi-liquid diet after surgery was  $2.5 \pm 0.7$  and  $4.1 \pm 1.3$  d, respectively. The average length of postoperative hospital stay was 6.2 d. Postoperative complications were observed in 12 (11.5%) patients and included anastomotic leakage in 1 (0.96%) patient, gastroplegia in 2 (1.9%) patients, prolonged ileus in 2 (1.9%) patients and poor wound healing in 2 (1.9%) patients. Postoperative complications are shown in Table 3. The anastomotic leak was mild and occurred in the duodenal stump. The patient recovered

**Table 1 Patient characteristics**

Characteristics (n = 104 cases)	
Age (yr, mean ± SD)	58.2 ± 12.6
Gender (male/female)	66:38
BMI (kg/m <sup>2</sup> , mean ± SD)	22.12 ± 4.64
ASA status	
I	28
II	72
III	4
Comorbidity	
Diabetes	14
Valvular heart disease	6
Chronic atrial fibrillation	4
Hypertension	26
Occlusive vascular disease	4
Chronic anemia	18
Primary bronchiectasis	2

BMI: Body mass index; ASA: American Society of Anesthesiologists.

**Table 2 Intraoperative data and early outcome (mean ± SD)**

Type of gastrectomy and anastomosis	
Total esophagojejunostomy with Roux-en-Y reconstruction	54
Distal Gastroduodenostomy/gastrojejunostomy	38
Proximal Esophagogastrotomy	12
TNM staging	
I	26
II	32
III	46
Operative time (min)	
Overall	272.52 ± 53.91
Total gastrectomy	302.5 ± 20.28
Distal subtotal gastrectomy	266.54 ± 35.26
Proximal gastrectomy	264.82 ± 40.33
Construction time (min)	45.8 ± 26.0
Total number of retrieved lymph node	23.1 ± 5.3
Estimated blood loss (mL)	80.78 ± 32.37
Hospital stay after surgery (d)	6.2 ± 2.5

fully following treatment with continuous irrigation drainage for 12 d. One patient underwent re-operation on post-operative day 14 due to jejunal afferent loop obstruction and recovered 10 d later. Another patient who underwent distal gastrectomy with gastroduodenostomy was readmitted due to intra-abdominal infection after surgery. She was treated with abdominal puncture and drainage and recovered.

## DISCUSSION

The first robotic cholecystectomy was performed by Cadière *et al*<sup>[19]</sup>. Currently, robotic surgery is widely applied in most operations. Recent studies have shown that robotic gastrectomy is feasible for patients with gastric cancer<sup>[20,21]</sup>.

A recent trend in minimally invasive surgery for the treatment of gastric cancer has attempted to reduce the length of the skin incision. The fact that minilaparotomy

**Table 3 Postoperative complications using the Clavien-Dindo classification**

Complications	Grade	Grade	Grade	Grade	Grade	Grade
	1	2	3a	3b	4	5
Anastomosis leakage	1					
Gastroplegia		2				
Prolonged ileus		2				
Alimentary tract obstruction				1		
Alimentary tract hemorrhage		1				
Poor incision healing			2			
Pulmonary infection		1			1	
Abdominal infection or abscess			1			
Intra-abdominal bleeding		1				
Total	1	7	3	1	1	

itself can cause traumatic stress to surgical patients led to the development of a totally laparoscopic technique in which all of the surgical procedures, including reconstruction, are performed intraabdominally under a laparoscopic field<sup>[22]</sup>. Various methods have been established to facilitate intracorporeal anastomosis<sup>[23-25]</sup>. A recent study in a large volume center showed that extracorporeal anastomosis can be changed to intracorporeal anastomosis using a stapling device. However, this laparoscopic method, especially in esophagojejunal anastomosis, presented many technical problems including exposure difficulty, impossible reinforced suturing, variation in the diameter of the esophagus and a weak point in double stapling<sup>[26-28]</sup>. Due to the technical difficulties of laparoscopic anastomosis and concern regarding anastomotic complications using the stapling method<sup>[29]</sup>, many surgeons still prefer extracorporeal reconstruction. However, with the advance of robotic surgery, the da Vinci system has become a minimally invasive cutting edge surgical technique. Since articulating instruments of the robotic device may provide complete wrist dexterity, allowing fine control with precision when performing intracorporeal sutures, a robot-sewn anastomosis in robotic gastric cancer surgery could avoid minilaparotomy and additional laparoscopic techniques, and provide surgeons with a reduced risk of anastomotic complications similar to hand sewing<sup>[22]</sup>.

Robotic operations improved the time to completion and the quality of choledochojejunostomy compared with laparoscopy in an *ex vivo* bench model, especially for surgeons with less experience with minimally invasive surgery<sup>[30]</sup>. Compared with standard laparoscopy, robotic assistance significantly improved intracorporeal suturing performance and the safety of novices in the operating room, thus significantly shortening the learning curve<sup>[31]</sup>. Three dimensional vision allows significant improvements in performance times and error rates for both inexperienced residents and advanced laparoscopic surgeons<sup>[32]</sup>. Hur *et al*<sup>[33]</sup> reported 2 cases of successful esophagojejunostomy using the full robot-sewn technique after total gastrectomy with lymph node dissection. The study further confirmed that all types of hand-

sewn anastomoses in gastrectomy, which are performed in the deep and narrow space of the abdominal cavity, were technically feasible<sup>[33]</sup>. Our study of 104 cases also demonstrated that full robotic hand-sewn anastomosis was technically feasible and safe.

Recent studies have demonstrated that the robotic approach does not provide an advantage over laparoscopy<sup>[34-37]</sup>. Twenty of the initial robot-assisted gastrectomies had similar results to those for experienced laparoscopically-assisted gastrectomies in one report. Other studies have shown that patients who undergo a robot-assisted gastrectomy have a larger number of dissected lymph nodes and a smaller amount of bleeding during radical surgery for early gastric cancer than those who undergo a laparoscopically-assisted gastrectomy<sup>[38,39]</sup>, but not in terms of hospitalization time after surgery<sup>[32]</sup>. Some scholars have indicated that one reason for the insufficient demonstration of this surgical system's advantages is that full robot-assisted reconstruction of the alimentary tract<sup>[40]</sup> was not performed in these studies. Before this clinical trial, we performed more than 100 cases of robot-assisted gastrectomy with minilaparotomy for anastomosis<sup>[41]</sup>. According to our experience, hospitalization time after robotic surgery with full intracorporeal anastomosis decreased by approximately 1 d compared to that with minilaparotomy for anastomosis. The rate of incision infection was sharply reduced in robotic surgery with full intracorporeal anastomosis. However, to achieve a definite result, a large number of robot-assisted gastrectomy cases and well-designed research are needed. As the number of robot-assisted gastrectomy cases increase, surgical outcomes may improve.

Although this trial showed many benefits in terms of clinical outcomes, limitations were still encountered. As a result of full robotic intracorporeal surgery, the tumor location may not be identified as easily as extra-corporeal anastomosis. Thus, preoperative examination with gastro-endoscopy and computed tomography is obligatory to determine the location of the tumor and the type of gastrectomy. As the specimen was extracted from the abdominal cavity through the extended intraumbilical port site, the stomach was opened to ensure the tumor margin was adequate. As in our study, 2 cases were diagnosed with early stage tumor in the middle part of stomach and the precise location of tumor was not palpable even with 3-D vision during surgery. Thus, these two cases were converted to extra-corporeal anastomosis *via* minilaparotomy to ensure adequate tumor margin.

The optimal method for full intracorporeal anastomosis remains to be established. It is probable that there is not one single optimal method. As we have shown, full robot hand-sewn anastomosis can be safely and rapidly performed by surgeons familiar with intracorporeal suturing and knot-tying techniques. This technique is feasible and can produce satisfying postoperative outcomes, and may be a minimally invasive technique in future gastrectomy surgery.

## COMMENTS

### Background

To achieve a minimally invasive method in gastrectomy surgery, a minimal gastroenteral anastomosis must be completed intracorporeally. Various modified procedures for reconstruction have been reported, but an optimal method has not been established due to technical difficulties. Robotic surgery has theoretical advantages such as increased degrees of freedom of instruments and a three-dimensional view. The aim of this study was to determine the feasibility and effectiveness of full robot-assisted total gastrectomy using intracorporeal robot hand-sewn anastomosis in the treatment of gastric cancer.

### Research frontiers

Hand-sewn suturing is technically demanding, but with the advantages of robotic surgery it can be performed safely by trained surgeons. This technique is feasible and can produce satisfying postoperative outcomes. Its convenience and reliability in anastomosis for gastrectomy were confirmed in the study. This is the first large scale report on full robot-assisted gastrectomy with intracorporeal robot-sewn anastomosis.

### Innovations and breakthroughs

The details of the surgical technique were well illustrated in this article. This technique is feasible and can produce satisfying postoperative outcomes, and may be a minimally invasive technique in future gastrectomy surgery.

### Applications

Intracorporeal robot-sewn anastomosis can be widely used in robotic surgery centers. It may be a minimally invasive technique in future robotic gastrectomy surgery.

### Terminology

Robotic surgery: Computer-assisted surgery and robotically-assisted surgery are terms used for technological developments which use robotic systems to aid surgical procedures. Robotically-assisted surgery was developed to overcome the limitations of minimally invasive surgery and to enhance the capabilities of surgeons performing open surgery. Minilaparotomy: A small abdominal incision for surgical procedures, such as liver biopsy, open transhepatic cholangiography, or alimentary anastomosis to ensure minimal traumatic stress.

### Peer review

Authors present their prospective experience with full robotic-assisted gastrectomy. They performed 104 successful operations ranging from distal gastrectomy with intracorporeal gastroduodenotomies or gastrojejunostomies to total gastrectomy with esophagojejunostomy. The average surgical time was 272 min and blood loss was 81 cc. Patients averaged 6.2 d in hospital. The authors conclude that robotic gastrectomy with intracorporeal anastomosis is feasible and safe. Further case-control studies need to be conducted to investigate the advantage of intracorporeal robot's hand sewn anastomosis.

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## HER2 in gastric cancer: Comparative analysis of three different antibodies using whole-tissue sections and tissue microarrays

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### Abstract

**AIM:** To compare the performance of three commercially available anti-human epidermal growth factor re-

ceptor 2 (HER2) antibodies in whole-tissue sections and tissue microarrays (TMAs) of a series of gastric tumors.

**METHODS:** We present a comparative analysis of three anti-HER2 antibodies (HercepTest, 4B5 and SP3) using TMA and whole-tissue sections prepared from the same paraffin blocks of 199 gastric adenocarcinomas operated upon between January 2004 and December 2008 at a Brazilian cancer hospital. The data on the patients' age, sex, the anatomical location of the tumor and the Lauren's histological classification were collected from clinical and pathological records. The immunohistochemical (IHC) results were examined by two pathologists and the cases were classified as positive (3+), equivocal (2+) and negative (0 or 1+), according to the criteria of the IHC scoring system of gastric cancer. TMAs and whole-tissue sections were evaluated separately and independently. All cases yielding discordant IHC results and/or scored as 2+ were subjected to dual-color *in situ* hybridization in order to determine the final HER2 status. Besides determining the sensitivity and predictive value for HER2-positive status, we measured the accuracy of each antibody by calculating the area under the receiver operating characteristic (ROC) curve. The agreement between the results obtained using the TMAs and those obtained using the whole-tissue sections was assessed by means of Kappa coefficient.

**RESULTS:** Intratumoral heterogeneity of HER2 expression was observed with all antibodies. HER2-positive expression (3+) in the whole-tissue sections was observed in 23 cases (11.6%) using the 4B5 antibody, in 18 cases (9.1%) using the SP3 antibody and in 10 cases (5.1%) using the HercepTest antibody. In the TMAs, 11 positive cases (5.6%) were identified using SP3 antibody, 9 (4.6%) using the 4B5 antibody and 6 (3%) using the

HercepTest antibody. The sensitivity using whole-tissue sections and TMA, respectively, was 95.2% and 42.9% with 4B5, 90.5% and 66.7% with SP3 and 47.6% and 42.9% with HercepTest. The accuracy, calculated from the area under the ROC curve, using whole-tissue sections and TMA, respectively, was 0.91 and 0.79 by 4B5, 0.86 and 0.80 by SP3 and 0.73 and 0.71 by HercepTest. The concordance of the results obtained using whole-tissue sections and TMA was 97.4% (Kappa 0.75) using HercepTest, 85.6% (Kappa 0.56) using SP3 and 84.1% (Kappa 0.38) using 4B5.

**CONCLUSION:** The use of the 4B5 antibody on whole-tissue sections was the most accurate IHC method for evaluating HER2 expression in gastric adenocarcinoma.

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**Key words:** Gastric cancer; Human epidermal growth factor receptor 2; Immunohistochemistry; Whole-tissue sections; Tissue microarray; Trastuzumab

**Core tip:** This is the first study to compare the three widely used anti-human epidermal growth factor receptor 2 (HER2) antibodies 4B5, SP3 and HercepTest in tissue microarrays and whole-tissue sections prepared from paraffin blocks of a single series of gastric tumors. We aimed to find the best method to assess HER2 expression in gastric cancer, facilitating the choice of the antibody with the greatest ability to identify the most patients who could benefit from the use of trastuzumab. Besides, we demonstrated that HER2 expression in small samples of gastric cancer (such as tissue microarrays and biopsies) should be evaluated cautiously because these tumors exhibit intratumoral heterogeneity that may influence the results.

Abrahão-Machado LF, Jácome AAA, Wohnrath DR, Santos JS, Carneseca EC, Fregnani JHTG, Scapulatempo-Neto C. HER2 in gastric cancer: Comparative analysis of three different antibodies using whole-tissue sections and tissue microarrays. *World J Gastroenterol* 2013; 19(38): 6438-6446 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6438.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6438>

## INTRODUCTION

The incidence of gastric cancer (GC) is gradually decreasing; however, it remains one of the leading causes of cancer-related death worldwide because the vast majority of GC patients are diagnosed with advanced disease<sup>[1-4]</sup>. Despite the improvement in surgical techniques and the use of multimodal treatments, the prognosis for GC is generally poor and treatment continues to be a challenge for physicians<sup>[1,4]</sup>. Recently, several oncogenes and tumor suppressor genes were studied in an attempt to clarify the process of gastric carcinogenesis, and specific monoclonal antibodies were developed as a potential form of adjuvant treatment for patients with advanced disease.

The HER2 (CerbB-2) or human epidermal growth factor receptor 2 (HER2) gene is a proto-oncogene located on chromosome 17q21 that encodes a transmembrane protein that is a member of the HER receptor family. These receptors possess tyrosine kinase activity and are typically involved in signal transduction pathways that lead to cell growth and differentiation<sup>[5]</sup>. Amplification of the *HER2* gene and overexpression of its product have been identified in several tumors and have been widely studied in breast cancer<sup>[6]</sup>. In GC, however, the reported frequency of HER2 overexpression ranges from 8.2% to 53.4%, and its clinical significance and prognostic value remain controversial, although HER2-positive tumors are usually associated with more aggressive biological behavior and tumor recurrence<sup>[7-13]</sup>. A recent meta-analysis showed that in 7 of the 15 papers evaluated, HER2 positivity was correlated with a worse prognosis<sup>[14]</sup>.

New advances in molecular targeting therapy have identified HER2 as an important target for anti-cancer therapy of gastric tumors. The ToGa study recently indicated improved survival of patients with advanced GC who were treated with trastuzumab (a chimeric anti-HER2 targeted drug) combined with chemotherapy compared with those treated with chemotherapy alone<sup>[15]</sup>. This randomized clinical trial achieved the longest median survival to date of patients with advanced gastric carcinomas. The mechanism by which trastuzumab acts is not completely understood, but the likely possibilities are that it prevents the dimerization of HER2 with other members of the HER family, activates the immune response by promoting antibody-dependent cell-mediated toxicity and induces endocytosis of HER2<sup>[16,17]</sup>. Given the demonstration of its clinical benefits and its approval for use in systemic therapy by the Food and Drug Administration (FDA), trastuzumab is the new standard treatment option for patients with HER2-positive advanced GC. Therefore, it is crucial to determine the HER2 status of GCs to select patients who may benefit from this promising targeted therapy.

Several assays are available to determine HER2 status; however, many of them require fresh tissue, involve complicated procedures and are costly. The most commonly used method is immunohistochemistry (IHC), which is a low-cost technique that can be performed on small samples, even formalin-fixed and paraffin-embedded tissues. Fluorescent *in situ* hybridization (FISH) is considered the gold standard and can be used to analyze this type of sample. However, because of its higher cost and the need for a fluorescence microscope, as well as the high concordance between FISH and IHC reported in literature<sup>[18-21]</sup>, generally only equivocal cases are subjected to FISH. An alternative for equivocal cases is provided by the use of other *in situ* hybridization methods such as silver *in situ* hybridization (SISH), including dual-color *in situ* hybridization (DISH), which allows the use of an ordinary light microscope and has shown excellent correlation with results obtained using FISH<sup>[18,22,23]</sup>.

Although a widely used and FDA/CAP-approved IHC scoring system already exists for HER2 in breast

cancer, it was necessary to develop a suitable scoring system for gastric tumors, mainly because of morphological differences and the intratumoral heterogeneity of HER2 expression in GC<sup>[8,9,11,18,24]</sup>. The system proposed by Hoffmann *et al*<sup>[9]</sup> for GC and incorporated as standard by CAP and FDA differentiates between surgical specimens and biopsies.

Currently, commercially available IHC antibodies include the HercepTest and A0485 (Dako, Glostrup, Denmark) rabbit polyclonal antibodies, the SP3 (Labvision; Thermo Fisher Scientific, Fremont, CA, United States) and 4B5 (Ventana Medical Systems, Tucson, AZ, United States) rabbit monoclonal antibodies and the CB11 mouse monoclonal antibody (Novocastra, Newcastle upon Tyne, England). Only the HercepTest, 4B5 and CB11 antibodies are approved by the FDA, although the international literature also shows high-quality of the SP3 antibody in samples of breast cancer<sup>[20,25]</sup>.

In the present study, HER2 expression in 199 GC was investigated by IHC on whole-tissue sections and tissue microarrays (TMA) using HercepTest, 4B5 and SP3. To date, no published results have compared these three antibodies. Moreover, this is the first study of GC to compare HER2 expression using both TMAs and whole-tissue sections prepared from samples of the same paraffin blocks, *i.e.*, the same tumors. All cases yielding divergent IHC results or results considered equivocal (2+) were subjected to DISH. We hypothesized that if the TMA samples were considered to be biopsies because they are small tissue samples, the reproducibility of the HER2 scoring system for GC could be tested using the two types of specimens.

Given that HER2 expression in the stomach is heterogeneous, the main purpose of our study was to compare the performance of three commercially available anti-HER2 antibodies. Furthermore, we aimed to determine the concordance of results obtained from whole-tissue sections and TMAs from the same tumors to evaluate the feasibility of TMA as an alternative method for assessing HER2 expression in GC.

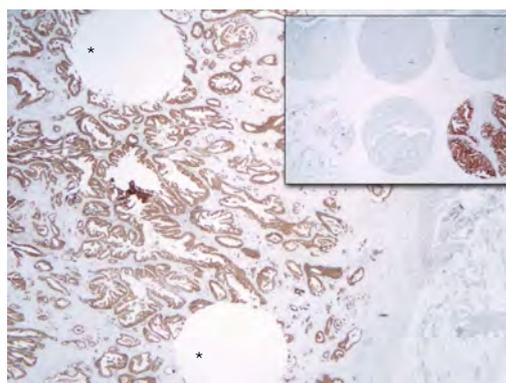
## MATERIALS AND METHODS

### Patients

In the present study, we selected 199 cases of surgically resected primary gastric or gastro-esophageal adenocarcinomas. All the patients were operated upon between January 2004 and December 2008 at the Barretos Cancer Hospital. Clinical data were collected from medical charts and pathology reports, including sex and patient age as well as the anatomical location of the tumor and its Laurén histological classification.

### TMA construction and IHC

Paraffin blocks containing representative samples of the tumors were selected by reviewing all of the hematoxylin and eosin (HE) stained slides. For the TMAs, two tissue cores with a diameter of 0.6 mm were extracted from

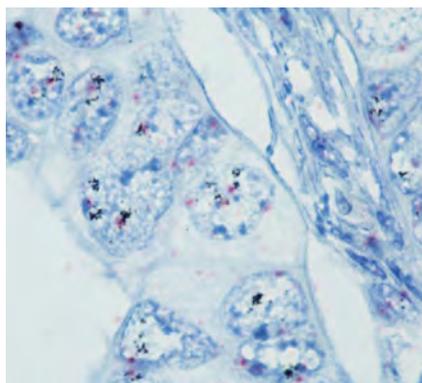


**Figure 1** Photomicrograph of immunohistochemistry ( $\times 100$ ) in a whole-tissue section. Asterisks indicate two round voids where the tissue microarray cores (inset) were extracted.

each tumor using the TMA arrayer MTA1 (Estigen, Tartu, Estonia). The tumor cores were sequentially placed in molds, embedded in paraffin and cooled to form the tissue array blocks. Each TMA also contained various non-gastric tissue samples as control tissues.

Sections of a thickness of 4  $\mu\text{m}$  were obtained from the whole-tissue paraffin blocks and TMA blocks and used for IHC (Figure 1). The slides were stained using automatic staining devices: the Benchmark XT (Ventana Medical Systems, Tucson, AZ) for the 4B5 and SP3 antibodies and the Autostainer Link 48 (Dako, Glostrup, Denmark) for the HercepTest. After antigen retrieval processing for 60 min (at pH 8.4), 4B5 (prediluted form as provided by the manufacturer) and SP3 (diluted 1:100) were applied for a 32 min incubation period. Antibody visualization was enabled using the Ventana Ultraview DAB detection kit. The HercepTest was performed according to the manufacturer's guide provided with the kit, using the prediluted "ready-to-use" form for all of the steps and incubation periods preprogrammed in the stainer software. The kit also contained the visualization reagent. All the slides were subsequently counterstained with hematoxylin.

The criteria suggested by Hoffmann *et al*<sup>[9]</sup> were used to evaluate the expression of HER2. Sections of the surgical specimens were considered HER2-positive (3+) when strong complete or basolateral membranous staining was detected in  $\geq 10\%$  of the neoplastic cells; equivocal (2+) when moderate/weak complete or basolateral membranous staining was detected in  $\geq 10\%$  of the cells; 1+ (negative) when the staining was weak or detected in only one part of the membrane in  $\geq 10\%$  of the cells and 0 (also negative) in cases in which there was no membranous staining or staining of  $< 10\%$  of the tumor cells. The criteria for evaluating biopsies were applied to the TMAs, and the percentage above (10%) was replaced by a cellular group of at least 5 cells. Full-tissue sections or TMA cores with excessive tissue fragmentation, scant invasive tumor and excessive cytoplasmic or background staining were rejected, and IHC was repeated on more suitable samples.



**Figure 2** Dual-color *in situ* hybridization (× 1000), human epidermal growth factor receptor 2 amplification. Black dots: Human epidermal growth factor receptor 2 gene; Pink dots: Chromosome 17.

### ***In situ hybridization***

DISH was performed in all cases that were scored 2+ in either of the samples stained with any of the antibodies, in accordance with the guidelines recommended by CAP and routine laboratory practice. In addition, all of the cases with discordant IHC results were tested by DISH. The tissue sections used for DISH were obtained from the whole-tissue paraffin blocks. HER2 DISH was performed using the Ventana Benchmark XT-machine (Ventana Medical System, Tucson, AZ) following its standardized protocol. DISH is dual-color *in situ* hybridization in which the *HER2* gene is labeled using silver to produce a black dot, and the centromere of chromosome 17 (Chr 17) is labeled with alkaline phosphatase to produce a pink dot. Therefore, the *HER2* gene and Chr 17 are both simultaneously stained on the same slide (Figure 2). The DISH slides were examined using an HE slide to assist with the tumor location and morphology within each section. At least 40 tumor cell nuclei were scored for the Chr 17 signal and HER2 signal in different areas of the tumor. Only nuclei displaying both signals were scored, and a HER2/Chr 17 ratio was obtained for each specimen. HER2 amplification was defined as a ratio of HER2/Chr 17  $\geq 2$ . Chromosome 17 polysomy was defined as  $\geq 3$  Chr 17 signals per cell on average<sup>[26]</sup>.

### ***Statistical analysis***

The statistical analysis was conducted using SPSS version 19.0 software (SPSS Inc., Chicago, IL). The IHC results were compared with a final variable of positivity or negativity for HER2 protein expression. In equivocal cases or cases of nonconcordant results obtained with the three antibodies, *HER2* gene amplification was assayed by DISH, and the DISH results determined the definitive HER2 status. Therefore, the results considered final, *i.e.*, the gold standard for statistical analysis, were those that were identical for the three antibodies, in addition to the results of DISH. The area under the receiver operating characteristic (ROC) curve (AUC) for each test was used to measure the accuracy of antibody labeling. To verify the agreement between the results obtained using the TMAs and those obtained using the whole-tissue sec-

tions, we employed the Kappa coefficient<sup>[27]</sup>. We defined  $P < 0.05$  as statistically significant.

## **RESULTS**

### ***Patients and tumor characteristics***

There were 123 male and 76 female patients, and their age ranged from 27 to 87 years (median: 60.7 years). The location of the tumor was in the antrum in 86 cases (43.2%), in the body in 26 cases (13%), in the fundus in 2 cases (1%), in the cardia in 38 cases (19%) and multicentric (in more than 2 regions) in 47 cases (23.6%). Fifty-five cases were histologically classified as the diffuse type (27.6%), 123 as the intestinal type (61.8%), 17 as the mixed type (8.5%) and four as not otherwise classified (2%).

### ***Immunohistochemistry***

HER2-positive expression (3+) in the whole-tissue sections was observed in 10 cases (5.1%) using the HercepTest, in 23 cases (11.6%) using the 4B5 antibody and in 18 cases (9.1%) using the SP3 antibody. Using the TMAs, 6 HER2-positive cases (3%) were identified using the HercepTest, 9 (4.6%) using the 4B5 antibody and 11 (5.6%) using SP3 antibody. The immunohistochemistry results are shown in Table 1.

The HercepTest demonstrated the lowest number of positive cases in both the whole-tissue sections and the TMAs. The SP3 antibody yielded the highest number of equivocal (2+) cases for both types of samples. The frequency of a score of 2+ was higher among the whole-tissue sections than among TMAs, except when using the HercepTest, which showed the opposite pattern.

The overall concordance between the results obtained using the TMAs and those obtained using the whole-tissue sections was 97.4% with the HercepTest, 84.1% with the 4B5 antibody and 85.6% with the SP3 antibody. According to the values of the Kappa coefficient, HercepTest provided substantial agreement between the TMAs and whole-tissue sections, the SP3 antibody provided moderate agreement and the 4B5 antibody provided fair agreement (Table 2).

Stronger membrane staining in positive cases was observed for the 4B5 antibody than for the other two antibodies (Figure 3). The diffuse cytoplasmic staining in the gastric foveolar epithelium and intestinal metaplasia that was observed when using 4B5 antibody was less pronounced when using the SP3 antibody and not observed when using the HercepTest antibody. Heterogeneous HER2 expression within the tumors was observed with all antibodies (Figure 4). All the positive cases were classified as intestinal type. Nuclear staining with the 4B5 and SP3 antibodies was observed in some of the diffuse adenocarcinomas.

### ***DISH and final HER2 status***

Cases with divergent results and those considered equivocal by IHC (scored as 2+) were subjected to DISH; there

**Table 1 Results of HER2 immunostaining using the three antibodies on whole-tissue sections and tissue microarrays *n* (%)**

Score	Whole-tissue sections			TMAs		
	HercepTest	4B5	SP3	HercepTest	4B5	SP3
0	179 (90.9)	125 (63.1)	128 (65.2)	185 (93.5)	174 (88.8)	162 (82.6)
1+	7 (3.5)	30 (15.2)	17 (8.5)	3 (1.5)	10 (5.1)	8 (4.1)
2+	1 (0.5)	20 (10.1)	34 (17.2)	4 (2.0)	3 (1.5)	15 (7.7)
3+	10 (5.1)	23 (11.6)	18 (9.1)	6 (3.0)	9 (4.6)	11 (5.6)
Total	197 (100.0)	198 (100.0)	197 (100.0)	198 (100.0)	196 (100.0)	196 (100.0)

TMA: Tissue microarray.

**Table 2 Concordance between the tissue microarrays and whole-tissue sections staining results using the HercepTest, 4B5 and SP3 antibodies**

Antibody	Overall concordance	Kappa coefficient (95%CI)
HercepTest	97.40%	0.75 (0.54-0.96)
4B5	84.10%	0.38 (0.22-0.53)
SP3	85.60%	0.56 (0.43-0.70)

were 58 (29.1%) such cases, of which 14 cases (24.1%) exhibited *HER2* gene amplification and 44 cases (76.9%) did not exhibit *HER2* gene amplification. Chr 17 polysomy was present in 5 cases (8.6%), but it was not related to amplification in our study. Table 3 shows the *HER2* gene status in the cases that presented an IHC score of 2+.

A final positive *HER2* status (either by IHC or DISH) was obtained in 20 of the 199 cases tested (10%). The sensitivity using whole-tissue sections was 47.6% with HercepTest, 95.2% with 4B5 and 90.5% with SP3 in cases with an immunoscore of 2+/3+. The sensitivity using TMA was 42.9% with HercepTest, 57.1% with 4B5 and 66.7% with SP3 (Table 4).

Table 5 demonstrates the accuracy of each antibody. The 4B5 and SP3 antibodies gave similar AUC values in whole-tissue sections (Table 5), and both were significantly more accurate than HercepTest ( $P = 0.002$  and  $0.035$ , respectively). Although the 4B5 and SP3 antibodies both gave greater AUC values than HercepTest in TMAs, the difference was not statistically significant. Based on the AUC of each antibody for both types of samples and the respective  $P$  values (Table 6), we determined that the use of the 4B5 antibody on whole-tissue sections was the most accurate method. SP3 staining of whole-tissue sections was also more accurate than the HercepTest using TMAs ( $P = 0.013$ ).

### Histological type and anatomical location

Of the 123 adenocarcinomas of the intestinal type, nine (7.3%) were given a final positive *HER2* status. None of the 76 cases of the other histological types was positive.

Of the 86 carcinomas located in the antrum, nine (10.4%) had a final positive *HER2* status. Eight (21%) of the 38 tumors situated in the cardia and three (6.4%) of the 47 multicentric tumors were positive.

## DISCUSSION

With the demonstration of the benefits of trastuzumab therapy for advanced GC<sup>[15]</sup>, the clinical demand for *HER2* assessment is rapidly increasing. The use of trastuzumab in association with platinum and capecitabine or 5-FU for *HER2*-positive GC has shown the longest median survival in GC patients<sup>[15]</sup>. Because IHC appears to be the easiest, least expensive and most widely used method, our goal was to compare three commercially available antibodies. Although the use of different clones can be problematic for GC, only two other reports in the literature have compared the performance of *HER2* antibodies<sup>[18,28]</sup>. An ideal antibody test would be sufficiently sensitive to identify all possible treatment candidates and would have a low false-positive rate to minimize over-treatment.

Variability in performance among commercially available anti-*HER2* antibodies has been demonstrated in several studies, although most of these studies were performed in breast tumors<sup>[29,30]</sup>. Cho *et al.*<sup>[28]</sup> compared the HercepTest, A0485, 4B5 and CB11 antibodies in TMAs of gastric carcinomas, and they found that the sensitivity and specificity were 78.9% and 96% with HercepTest, respectively, 86.5% and 94.4% with the A0485 antibody, 76.3% and 95.6% with 4B5 and 60.5% and 98.4% with the CB11 antibody. Boers *et al.*<sup>[18]</sup> tested the SP3 and 4B5 antibodies on biopsy specimens of gastro-esophageal adenocarcinomas, and they showed sensitivities of 77% and 96% as well as specificities of 100% and 98.4%, respectively. The latter result is consistent with the results we obtained when comparing the SP3 and 4B5 antibodies. In the present study, however, the difference in sensitivity among antibodies was much higher, as the sensitivity ranged from 47.6% to 95.2% when whole-tissue sections were analyzed and ranged from 42.9% to 66.7% when TMAs were analyzed. The sensitivity of HercepTest was far lower than that of the other two tests, which contributed to this difference. The specificity ranged from 81.2% to 99.4% for the whole-tissue sections and from 93.1% to 100% for the TMAs. For all the antibodies, lack of staining (score 0) was highly predictive of a negative/nonamplified case as confirmed by DISH, and positive staining (score 3+) was highly predictive of an amplified case as confirmed by DISH.

The 4B5 and SP3 antibodies exhibited similar perfor-

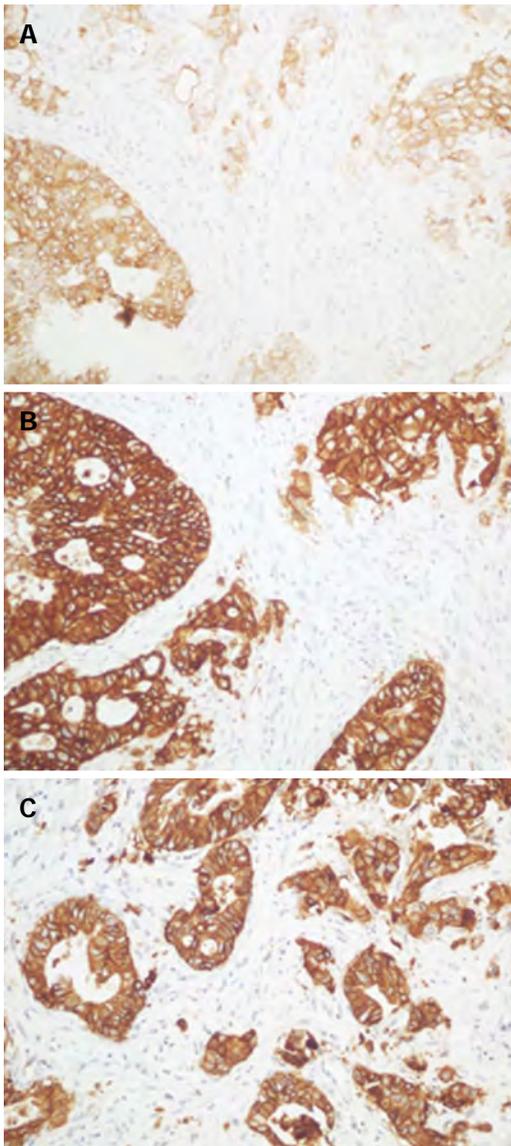


Figure 3 Comparison of positivity (3+) using the HercepTest (A), 4B5 (B) and SP3 (C) antibodies ( $\times 200$ ).

mance, with high NPV values and AUC values that indicated higher accuracy, compared to HercepTest, although the difference was only statistically significant ( $P < 0.05$ ) for whole-tissue sections. Even though HercepTest had high values for PPV and specificity, it presented the lowest sensitivity. Thus, this antibody provided the highest number of tumors with immunoscores of 0 or 1+ that were positive for HER2 amplification using DISH, which agrees with the report of Dekker *et al.*<sup>[29]</sup> for breast tumors. Thus, in our view, HercepTest is not the best antibody to use as a first-line test to assess the HER2 status of GC because the ideal antibody should be highly sensitive, even though high sensitivity could increase the number of equivocal cases (2+) and the need for *in situ* hybridization tests. The 4B5 and SP3 antibodies were highly sensitive; therefore, these two antibodies appear to be more reasonable for first-line tests than HercepTest.

Another issue that we wanted to address was the use

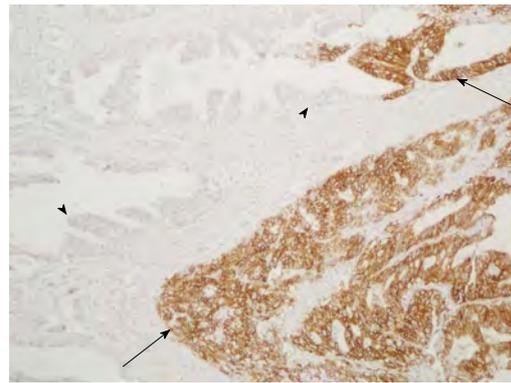


Figure 4 Representative image of the intratumoral heterogeneity of HER2 expression ( $\times 100$ ). Arrows indicate areas with strong continuous membranous staining (score 3+) and arrowheads indicate negative areas (score 0).

of TMAs for assessment of HER2 and, by analogy, the reliability of testing endoscopic biopsies. The use of TMAs permits the inclusion of several different tumors in the same assay on a single slide. This cost-effective technique has become a standard procedure for many contemporary IHC studies. Dekker *et al.*<sup>[29]</sup> found that TMAs were reliable for retesting large volumes of previously HER2-classified breast carcinomas. Similarly, Drev *et al.*<sup>[31]</sup> observed a high concordance between whole-tissue sections and TMAs for breast tumors. Despite these favorable results for TMAs of breast cancers, HER2 assessment of gastric adenocarcinomas is more problematic. The obvious disadvantage of TMAs is that this preparation enables the analysis of only a limited sample of the tumor and for GCs, TMAs are even more unfavorable because of the generally observed heterogeneous expression of HER2 within these tumors.

Intratumoral heterogeneity can be defined as areas with different HER2 scores within the same tumor. This is the predominant pattern for GCs but not for breast tumors, and may thus cause sampling error when randomly sampled TMA cores of GCs are used<sup>[8,9,11,24]</sup>. The difference in HER2 status between primary and metastatic tumor samples is still a matter of debate, and although the few studies in the scientific literature have demonstrated high concordance among the results obtained using these samples, some cases gave discordant results<sup>[32,33]</sup>, which suggests an effect of intratumoral heterogeneity.

Conspicuously heterogeneous HER2 staining on the whole-tissue sections and TMA core samples from most of the tumors was noted in our study. To minimize the discrepancy with results obtained with whole-tissue sections, we included two core samples from different areas of each tumor in the TMAs. Regardless, the TMA staining was much less sensitive than the staining of whole-tissue sections (mean values for the antibodies: 55.5% *vs* 77.7%, respectively). Although the HercepTest provided greater agreement between the TMAs and the whole-tissue sections, with a substantial Kappa value, its sensitivity was low for both types of sample, as mentioned above. The difference in HER2 expression detected in

**Table 3** Human epidermalgrowth factor receptor 2 gene status assessed by dual-color *in situ* hybridization in cases with an immunohistochemistry score of 2+ with HercepTest, 4B5 and SP3 staining on whole-tissue sections and tissue microarrays *n* (%)

HER2 gene status	Whole-tissue sections			TMAs		
	HercepTest	4B5	SP3	HercepTest	4B5	SP3
Amplified	0 (0)	6 (30)	6 (17.6)	3 (75)	3 (100)	4 (26.6)
Not amplified	1 (100)	14 (70)	28 (82.4)	1 (25)	0 (0)	11 (73.4)
Total	1 (100)	20 (100)	34 (100)	4 (100)	3 (100)	15 (100)

TMA: Tissue microarray; HER2: Human epidermalgrowth factor receptor 2.

**Table 4** Specificity, sensitivity, positive and negative predictive values and the area under the receiver operating characteristic curve of each antibody according to the final human epidermalgrowth factor receptor 2 status

		HercepTest (95%CI)	4B5 (95%CI)	SP3 (95%CI)
TMA	Sensitivity	42.9 (21.7-64.0)	57.1 (35.9-78.3)	66.7 (46.5-86.8)
	Specificity	99.4 (98.3-100.0)	100.0	93.1 (89.4-96.9)
	PPV	90.0 (71.4-100.0)	100.0	53.8 (34.7-73.0)
	NPV	93.6 (90.1-97.1)	95.1 (92.0-98.2)	95.9 (92.9-98.9)
	AUC	0.71 (0.60-0.782)	0.79 (0.68-0.89)	0.80 (0.69-0.90)
Whole-tissue sections	Sensitivity	47.6 (26.2-68.9)	95.2 (86.1-100.0)	90.5 (77.9-100.0)
	Specificity	99.4 (98.3-100.0)	87.0 (82.0-91.9)	81.2 (75.5-87.0)
	PPV	90.9 (73.9-100.0)	46.5 (31.6-61.4)	36.5 (23.4-49.6)
	NPV	94.1 (90.7-97.5)	99.3 (98.1-100.0)	98.6 (96.7-100.0)
	AUC	0.73 (0.63-0.84)	0.91 (0.86-0.96)	0.86 (0.78-0.93)

PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the receiver operating characteristic curve; TMA: Tissue microarray.

**Table 5** Accuracy of the three antibodies in the whole-tissue sections and the tissue microarrays

		HercepTest	4B5	SP3
Whole-tissue sections	AUC	0.73	0.91	0.78
	HercepTest	-	<i>P</i> = 0.002	<i>P</i> = 0.035
	4B5	<i>P</i> = 0.002	-	<i>P</i> = 0.265
TMAs	AUC	0.71	0.79	0.8
	HercepTest	-	<i>P</i> = 0.058	<i>P</i> = 0.075
	4B5	<i>P</i> = 0.058	-	<i>P</i> = 0.714
	SP3	<i>P</i> = 0.075	<i>P</i> = 0.714	-

AUC: Area under the receiver operating characteristic curve; TMA: Tissue microarray.

**Table 6** Comparison of the areas under the receiver operating characteristic curve according to the type of sample (whole-tissue section and tissue microarray) and the antibodies

		Whole-tissue sections			
		HercepTest	4B5	SP3	
TMAs	AUC	0.73	0.91	0.78	
	HercepTest	0.71	<i>P</i> = 0.572	<i>P</i> = 0.001	<i>P</i> = 0.013
	4B5	0.79	<i>P</i> = 0.289	<i>P</i> = 0.027	<i>P</i> = 0.201
	SP3	0.80	<i>P</i> = 0.265	<i>P</i> = 0.034	<i>P</i> = 0.244

AUC: Area under the receiver operating characteristic curve; TMA: Tissue microarray.

our study between TMAs and whole-tissue sections was caused by the prominent heterogeneity of HER2 staining. The 4B5 antibody results in whole-tissue sections were significantly different from the results of the three antibodies in TMAs. Because 4B5 antibody staining of whole-tissue sections had the highest accuracy, which was much different from that obtained for TMAs, our results suggest that TMA staining is less accurate and lacks sufficient sensitivity to reliably assess the HER2 status in GC. Two tissue cores for the TMA were definitely not sufficient to prevent sampling error and minimize false results because of the intratumoral heterogeneity and the small amount of tissue in the cores. Therefore, studies in the literature that used TMA to test the HER2 status of GCs must be carefully analyzed, and it must be noted that this technique does not seem to reflect the real status of the HER2 gene in GCs.

Endoscopic biopsies with few fragments, such as those used in the TMA, may underestimate the incidence of HER2-amplification, as Yang *et al*<sup>[24]</sup> demonstrated in a recent study in which large surgical specimens had higher rates of HER2 positivity than biopsy specimens. We believe that endoscopic biopsies are not optimal to identify the maximal number of patients who could be eligible for treatment. Therefore, to represent the tumor better and reduce misinterpretation, it is important to examine as many pieces of a biopsy as possible. We also suggest that all excisional specimens that had a previous HER2-negative result in a biopsy specimen should be retested to increase the chance of classifying the tumor as HER2-positive. Because the intratumoral heterogeneity of HER2 expression also seems to cause divergent results for primary and metastatic tumor samples<sup>[32]</sup>, is highly advisable to analyze the HER2 status of both primary and metastatic specimens when possible.

Thus, among the HercepTest, 4B5 and SP3 antibodies, HercepTest was the least sensitive and therefore had the lowest ability to identify a large number of patients eligible for trastuzumab treatment. According to our results, the most accurate IHC method to assess HER2 expression in GC is the use of the 4B5 antibody on whole-tissue sections. Intratumoral heterogeneity appears to be a major limitation for the use of TMA because TMA does not reflect the true HER2 status of many tumors. Because the number of cells that respond to a targeted therapy directly affects the tumor's responsiveness to treatment, there must be a great difference between cases that are diffusely positive and those that are only focally positive but still meet the criteria of positivity. Given the promising results from the use of trastuzumab and the particularities of HER2 expression in the stomach, further trials are needed to determine the clinical significance of the intratumoral heterogeneity and its impact on treatment outcome.

## COMMENTS

### Background

The vast majority of patients with gastric cancer are diagnosed with advanced disease and the prognosis is generally poor. Human epidermal growth factor receptor 2 (HER2)-positive tumors are usually associated with more aggressive biological behavior and recurrence. In view of the recently demonstrated clinical benefit of the anti-HER2 drug trastuzumab in the treatment of advanced gastric cancer, reliable HER2 testing is of key importance. HER2 status is usually determined by immunohistochemistry (IHC) and occasionally by *in situ* hybridization (ISH). However, little is known regarding the performance difference among the commercially available anti-HER2 immunohistochemical antibodies in gastric adenocarcinomas. This study compared three anti-HER2 antibodies (HercepTest, 4B5 and SP3) using two different arms: samples prepared in tissue microarray (TMA) device and whole-tissue sections counterpart prepared from paraffin blocks of a series of 199 gastric adenocarcinomas.

### Research frontiers

HER2 is a member of the family of tyrosine kinase receptors. Overexpression of the HER2 receptor has been identified in various cancers and is most widely studied in breast cancer. Like in breast cancer, HER2-positive gastric tumors are correlated with worse prognosis. An important difference in HER2 immunoreactivity between breast cancer and gastric adenocarcinomas is the striking heterogeneity of HER2-positivity in the latter, which can affect the determination of HER2 status in small samples such as biopsies and TMA.

### Innovations and breakthroughs

Many studies have compared anti-HER2 antibodies in breast cancer, however only few reports have done it in gastric cancer. Gastric adenocarcinomas exhibit intratumoral heterogeneity of HER2 expression and have a unique IHC scoring system. This is the first study to compare HercepTest, 4B5 and SP3 in a series of gastric tumors. Most contemporary studies use the cost-effective TMA technique for testing HER2 expression; however, given the high incidence of heterogeneous HER2-immunoreactivity and the risk of underestimating the incidence of HER2-amplification rate; we also aimed to compare the results obtained from TMA to those obtained from whole-tissue sections.

### Applications

The study results indicate the best method to assess HER2 expression in gastric cancer, facilitating the choice of the antibody with the greatest ability to identify the most patients who could benefit from the use of trastuzumab. The results also demonstrated that HER2 expression in small samples of gastric cancer should be cautiously evaluated because the intratumoral heterogeneity may influence the results.

### Terminology

According to the four-tiered IHC scoring system for gastric cancer, samples scored as 0 and 1+ are negative, 2+ as equivocal and 3+ as positive. Intratumoral heterogeneity is defined as areas with different HER2 scores within the

same tumor.

### Peer review

This is a highly stringent study in which the authors examined HER2 immunostaining in a series of gastric cancers, using three different commercially available anti-HER2 antibodies in samples of TMA and whole-tissue sections. The sensitivity, predictive value for HER2 amplification and accuracy of each antibody were determined. The results are provoking and indicate the most accurate IHC method for HER2 evaluation, emphasizing the limitations of the TMA technique. The results of the study should encourage a more judicious assessment of the HER2 status in gastric cancers, especially in small tissue samples.

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## Emergency admissions due to swallowed foreign bodies in adults

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### Abstract

**AIM:** To study a retrospective analysis of patients who presented to the emergency departments (ED) with complaints related to foreign body ingestions.

**METHODS:** Patients older than 16 years of age who presented to the ED between January 1<sup>st</sup> and December 31<sup>st</sup> of 2010 with complaints related to swallowed foreign bodies were identified from electronic health records and patient charts.

**RESULTS:** A total of 100 patients presented with a complaint of foreign body ingestion during the study period. Overall, an X-ray was performed on 75 patients, and a fiberoptic evaluation was performed on 45 patients. A foreign body was detected in 46 (46%) patients. The diagnostic yield of the X-ray was 27 (36%)

out of 75 patients, while the diagnostic yield of the fiberoptic evaluations was 21 (47%) out of 45 patients. The detected foreign bodies were mostly located in the esophagus (17 out of 46 foreign bodies detected). When the types of ingested foreign bodies were evaluated, 52 (52%) patients reported ingesting food, and 19 (19%) patients reported swallowing pins. An X-ray was performed on 33 patients with accidental food ingestions but yielded a positive result in only two cases. In 12 out of 21 patients with accidental food ingestion who underwent fiberoptic evaluation, the foreign material was detected and removed.

**CONCLUSION:** Plain radiography is helpful in the localization of radiopaque swollen foreign bodies, while fiberoptic methods are useful as both diagnostic and therapeutic tools, regardless of radiopacity.

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**Key words:** Foreign body; Ingestion; Gastrointestinal tract; Endoscopy; Emergency

**Core tip:** The majority of foreign bodies swallowed by patients who present to the emergency departments cannot be detected using standard imaging studies and evaluation. Plain radiography is especially useful in the localization of radiopaque foreign bodies, while fiberoptic methods can be used as both diagnostic and therapeutic tools, regardless of the radiopacity of the foreign body ingested.

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## INTRODUCTION

Visits related to gastrointestinal foreign bodies are relatively common causes of admission to emergency departments (ED)<sup>[1,2]</sup>. The ingestion or insertion of a foreign body into the gastrointestinal (GI) tract can be a clinically serious condition with associated risks for morbidity and mortality<sup>[2,3]</sup>. An estimated 1500 to 1600 patients die in the United States each year as a result of complications related to the ingestion or insertion of foreign bodies into the GI tract<sup>[1,3-5]</sup>. Although this problem can be encountered in every age group, almost 80% of cases comprise patients in early childhood (18-48 mo), with a majority of cases resulting from swallowing coins, toys, crayons, or pen caps<sup>[3,4]</sup>. The ingestion of foreign bodies is rarely seen in adults, is generally accidental and is commonly seen in the form of food (meat and bones) ingestion. Other risk groups for this type of injury include patients with psychiatric disorders, adults without teeth, prisoners and patients under the influence of substances that obscure judgment<sup>[3,5-7]</sup>. The clinical presentation, symptoms and management of foreign bodies depend on their location within the GI tract. Depending on the size and shape, almost 80%-90% of such foreign bodies pass freely from the GI tract without any complication<sup>[4,7,8]</sup>.

The purpose of the present study was to conduct a retrospective analysis of patients who presented to our ED with complaints related to foreign body ingestions.

## MATERIALS AND METHODS

Patients older than 16 years of age who presented to the Emergency Department between January 1<sup>st</sup> and December 31<sup>st</sup> of 2010 with complaints related to swallowed foreign bodies were analyzed retrospectively using the data obtained from electronic health records and patient charts. The patients' present complaints, demographic characteristics, previous medical history and medication use, physical exam findings, diagnostic studies performed, type and location of the foreign body, treatment provided, need for conservative or invasive/surgical treatment, complication rates, radiological findings and rate of survival/mortality were all recorded.

## RESULTS

During the study period, we identified a total of 100 patients (42 male, 58 female; mean age 38 years, range 16-88 years) who were admitted with a complaint of foreign body ingestion. Of those, 65 (65%) localized their complaints to the pharynx, while 35 (35%) told us that they had ingested the foreign bodies. Among the list of complaints, 53 (53%) patients had difficulty swallowing; 33 (33%) had pain in the throat; 6 (6%) had difficulty breathing; 5 (5%) had abdominal pain; 4 (4%) had vomiting; 4 (4%) had bleeding from the mouth; 2 (2%) had a foreign body sensation in the throat; 2 (2%) had coughing; and 1 (1%) had chest pain. The incident was self-reported as

accidental in all patients. When facilitating factors were considered, 3 (3%) patients were undergoing dental interventions, and another 3 (3%) patients had dental plates. None of the study patients had any established diagnosis of psychiatric disease or history of substance abuse, alcohol or sedative use. Physical examination revealed oropharyngeal foreign bodies in 7 patients, epigastric tenderness in 1 patient, and rhonchus in 1 patient.

The diagnostic approaches to our patients are summarized in Figure 1. A foreign body was detected in 46 (46%) patients. The diagnostic yield of X-rays was 27 (36%) among the 75 patients evaluated by lateral neck, chest or abdominal X-ray. The foreign bodies were detected for 10 out of 51 patients using the chest X-ray, for 14 out of 29 patients using the abdominal X-ray and for 3 out of 52 patients using the lateral neck X-ray. The diagnostic yield was 21 (47%) out of 45 for all patients undergoing fiberoptic evaluations (Figure 1). The detected foreign bodies were mostly located in the esophagus (17 out of 46 foreign bodies detected) (Table 1). When the types of ingested foreign bodies were evaluated, 52 (52%) patients reported ingesting food, and 19 (19%) patients reported swallowing pins (Figure 2, Table 2). With respect to the types of ingested food, 20 were fish bones, 9 were bone fragments (Figure 3), and 23 were unknown food parts.

In 53 (53%) of the patients, a conservative approach for management was considered. Nineteen (19%) patients were followed with serial radiological examinations. In 21 patients, of whom 17 were undergoing upper GI endoscopy, 2 were undergoing laryngoscopy and 2 were undergoing bronchoscopy, the foreign body was removed by fiberoptic means. In total, 19 (19%) of the study patients were admitted for further evaluation and treatment. Out of all the patients, the clinical course was complicated by aspiration (food material) in two patients, by GI bleed (pin) in 1 patient and by mediastinitis (food material) secondary to perforation in 1 patient.

## DISCUSSION

The medical history obtained from the patient is highly critical in the diagnosis of swollen GI foreign bodies. Therefore, the planning of the diagnostic work-up and the extent and urgency of a possible intervention are decided according to the information provided by the patient regarding the type of foreign body ingested, together with the clinical complaints and physical examination<sup>[6,9-12]</sup>. Most GI foreign-body ingestions occur in pediatric patients aged between 6 mo and 6 years<sup>[5,7,9]</sup>. GI foreign body exposure tends to be accidental in adults, with food particles and bones constituting the majority of the foreign bodies<sup>[4,13]</sup>. The rest of the cases occur in the setting of facilitating factors, such as adults without teeth or with dental plates, prisoners and psychiatric patients<sup>[4,6,9,14,15]</sup>. Our results were also similar among all of the patients evaluated in the study reporting accidental intake. Patients who suffer foreign body ingestion can present with a wide range of symptoms, which can vary

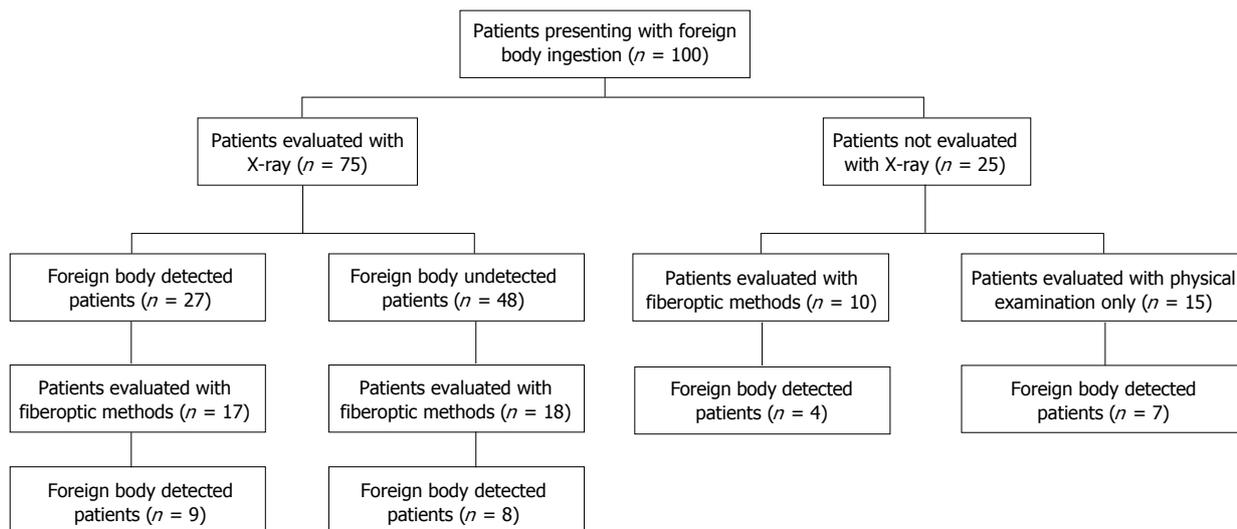


Figure 1 Diagnostic approach to patients presenting with foreign body ingestions.

Table 1 Location of the foreign bodies (n = 100)	
Foreign body location	n (%)
Esophagus	17 (17)
Oropharynx	8 (8)
Small intestine	6 (6)
Stomach	6 (3)
Trachea	2 (2)
Larynx	3 (3)
Colon	3 (3)
Undetermined location	1 (1)
Undetected	54 (54)

based on the physical characteristics and the content that is absorbed in the GI tract. Diagnosis is based on the patient’s history and complaints, which typically include the sudden onset of difficulty of swallowing during eating, chest pain, odynophagia or insufficiency in tolerating secretions. However, symptoms range from mild to life-threatening, including shortness of breath, abdominal pain, vomiting, hematemesis, foreign body sensation, coughing and chest pain<sup>[3,5,9,11,16,17]</sup>. In agreement with the literature, the majority of patients in our cohort presented with difficulty in swallowing and foreign body sensations in the throat.

Different types of foreign bodies are observed in the GI tract based on the age group. During childhood, swallowed coins, small toys, crayons or batteries are observed, whereas during adulthood, food, bones and dental-related foreign bodies are more common<sup>[4,6,7,9,13,16,17]</sup>. The types of foreign bodies may also differ by country. The high number of pin ingestions in our study group is thought to be related to the regional dress code, which results in women holding pins between their lips before attaching their headscarves. While certain conditions, such as parental attitudes and dietary habits, can provide clues for the types of foreign bodies that are ingested, prevention strategies are also dependent on various cultural, social,

religious and economic factors<sup>[7,11,17-19]</sup>.

The presentation, clinical findings, and management of foreign bodies are distinct and based upon the anatomical region where the foreign body is located<sup>[4,9,11,17]</sup>. Determining the type and location of the foreign body in the ED changes the treatment approach<sup>[5,9,16]</sup>. In our study, most of the foreign bodies were detected in the upper GI tract. The majority of the radiopaque foreign bodies in the GI tract can be detected using radiography. This simple modality provides crucial information, such as the number, size, location and direction of the foreign body, as well as the presence of sharp edges<sup>[2,3,6,8]</sup>. However, the presence of fish bones, chicken bones, glass, wood and thin metals cannot be ruled out by plain radiographies<sup>[2,3,6,11,13,20]</sup>. Neck, chest and abdominal radiographies are able to show perforations as well as metal objects and bones<sup>[6,13]</sup>. In our study, we detected a foreign body with plain radiography in 27 (36%) out of 75 patients evaluated by X-rays; all of these foreign bodies were radiopaque. All of the 19 patients with radiopaque foreign bodies in whom an emergency intervention was not planned were admitted for serial radiographic evaluations to determine the passage of the foreign body. Serial radiographic studies can be used to determine the passage of the foreign body and the complications resulting from it. If perforation is suspected based on the clinical or radiological findings, neck, chest or abdominal computed tomography is then indicated<sup>[13]</sup>. Computed tomography (CT) is especially useful when radiolucent materials cannot be detected with plain X-rays. A three-dimensional reconstruction with CT also increases the sensitivity of the detection modality<sup>[2,6,21-23]</sup>. CT can also be useful in determining, treatment options and complications.

Foreign bodies in the GI tract are typically treated conservatively, based on the type of foreign body and the patient’s clinical condition. Between 80% and 90% of foreign bodies pass through the GI tract freely, while 10% to 20% require an endoscopic intervention, and 1%



Figure 2 Plain abdominal X-ray showing a pin (white arrow) in the bowel in an adult. The pin passed spontaneously.



Figure 3 Lateral neck X-ray showing a bone fragment (white arrow). The fragment was removed by fiberoptic means.

**Table 2 Types of foreign bodies swallowed**

	Foreign body type	Total detected foreign bodies	Patients underwent X-ray	Foreign bodies detected with X-ray	Patients who underwent fiberoptic evaluation	Foreign bodies detected with endoscopy
	<i>n</i>	<i>n</i>	<i>n</i>	<i>n</i>	<i>n</i>	<i>n</i>
Food	52	20	33	2	21	12
Pin	19	17	19	17	10	6
Toothpick	5	1	3	-	4	1
Dental instrument	5	2	5	2	2	-
Tooth filling	4	2	4	2	2	1
Nail	2	2	2	2	2	1
Water gel beads (pearl shape crystal soil)	1	-	1	-	-	-
Gelatin paper	1	-	-	-	1	-
Pen cap	1	-	-	-	1	-
Earring	1	-	1	-	1	-
Coin	1	1	1	1	-	-
Medication	2	-	1	-	-	-
Chewing gum	1	1	1	1	1	-
Unknown	5	-	4	-	-	-
Total	100	46	75	27	45	21

require surgery<sup>[4,6-9,11,13,17,24,25]</sup>. Physicians should determine if and when an intervention is needed. The patient management strategies depend on a patient’s age and clinical condition, the type and size of the foreign body, the presence of sharp edges, the anatomical location and the endoscopic capability of the treating unit<sup>[2,3,5]</sup>. In general, foreign bodies larger than 2.5 cm in diameter cannot pass the pylorus, while objects longer than 6 cm cannot pass the duodenal curve. Therefore, these objects require endoscopic removal<sup>[2,6,19,26]</sup>. Endoscopic intervention is also indicated if the patient’s condition is not stable or if the foreign body is impacted or presents risks of further damage to the patient<sup>[13,27]</sup>. An emergent endoscopic removal should be performed in patients with esophageal obstruction (*e.g.*, cannot swallow secretions), those with disc batteries in the esophagus and those who have swallowed pointed objects<sup>[6,13]</sup>. However, endoscopic removal is contraindicated if the foreign body is above the upper esophageal sphincter or if there is clinical or radiological evidence of perforation. Objects containing illegal drugs must be removed with endoscopy, but this technique should be avoided in cases where ruptured cocaine pack-

ages are present in the GI tract<sup>[6,13]</sup>. Objects located proximal to the upper esophageal sphincters are suggested to be removed by ear-nose-throat specialists<sup>[6,13]</sup>. Emergent endoscopy should be performed to remove magnets if they are within the reach of the technique<sup>[6]</sup>. Monitoring of the spontaneous passage of coins in asymptomatic patients is recommended. If there is no spontaneous passage, then removal within 24 h of ingestion is recommended<sup>[6,9]</sup>. We conducted conservative monitoring in slightly over half of our patients (53 out of 100). The diagnostic yield was 21 (47%) out of 45 for all patients undergoing fiberoptic evaluations. Endoscopic treatment options for meat or other food impactions included food extraction and advancement of the bolus into the stomach<sup>[6,9]</sup>. In our study, an X-ray was performed on 33 patients with accidental food ingestions but yielded a positive result in only two cases. By contrast, among 21 patients with accidental food ingestion who underwent fiberoptic evaluation, 12 patients were found to have a foreign material, and the material was removed. Food ingestion, a subjective feeling of foreign body sensation, and other properties of foreign bodies may have resulted

in the poor yield of standard imaging studies and endoscopic and physical examinations<sup>[11,27]</sup>.

An alternative radiological tool that was not systematically assessed in our study was ultrasonography, which is uncommonly used to diagnose GI foreign bodies in adults<sup>[28]</sup>. However, there are reports in the literature where ultrasonography proves to be useful in the detection of abdominal foreign bodies<sup>[9,29]</sup>. However, abdominal ultrasonography can be used as an initial imaging modality in the diagnosis of GI foreign bodies in pediatric patients<sup>[30]</sup>.

Conditions such as acute abdomen due to intestinal perforations are seen in nearly 1% of patients who have ingested foreign bodies<sup>[8,31]</sup>. This condition can lead to severe complications and even death<sup>[32]</sup>. The most common complication of foreign body ingestion is perforation<sup>[33]</sup>; the ingestion of a sharp and pointed object is more likely to cause perforations<sup>[9,33,34]</sup>. Approximately 30%-35% of such objects can penetrate the GI tract, requiring endoscopic management<sup>[35]</sup>. In the presence of complications or in the case of unsuccessful endoscopic interventions, emergency surgery is preferred<sup>[35,36]</sup>. The majority of foreign bodies pass through the GI tract freely, without any complication, and only a small percentage of these cases require intervention<sup>[4,9]</sup>.

In conclusion, a majority of the swallowed foreign bodies in patients presenting to the ED cannot be detected using standard imaging studies and evaluation. Plain radiography is especially useful in the localization of radiopaque foreign bodies, while fiberoptic methods can be used both as diagnostic and therapeutic tools, regardless of the radiopacity of the foreign body ingested. The goal of ED management is to refer patients with clinical significance to the appropriate departments for further evaluation and treatment. It is therefore important to evaluate the type and location of the foreign body and to identify complications that might develop as a result of this entity.

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## COMMENTS

### Background

Ingestions or insertions of foreign bodies are rarely seen in adults, are generally accidental and are commonly seen in the form of food ingestion (such as meat and bones). Nonetheless, foreign body ingestions into the gastrointestinal tract can lead to clinically serious conditions with significant morbidity and mortality. Approximately 80%-90% of the foreign bodies pass through the gastrointestinal tract freely, while 10%-20% require an endoscopic intervention, and 1% require surgery.

### Research frontiers

The majority of swallowed foreign bodies in patients presenting to emergency departments cannot be detected using standard imaging tools. The research hotspot discussed here is that fiberoptic methods were implemented in both the diagnosis and treatment of gastrointestinal foreign bodies.

## Innovations and breakthroughs

The most common approach for the detection of ingested foreign bodies in the gastrointestinal tract is physical examination combined with radiological studies. While radiological examinations are especially helpful in the detection of radiopaque foreign bodies, these studies are unyielding in a significant proportion of patients, depending on the timing of presentation and the nature of the ingested foreign body. Early evaluation with fiberoptic methods helps not only in the diagnosis and localization of the foreign body but also in its removal.

## Applications

Dietary habits and various cultural factors can provide clues for the types of foreign bodies likely to be ingested. Fiberoptic methods can be used to diagnose and treat accidental foreign body ingestions in an emergency department setting.

## Peer review

The present study is a retrospective analysis of 100 patients with complaints related to foreign body ingestions. The data suggest that plain radiography is especially useful in the localization of radiopaque foreign bodies, while fiberoptic methods can be used as both diagnostic and therapeutic tools, regardless of the radiopacity of the foreign body ingested. This study highlights interesting points regarding the clinical treatment of complaints related to foreign body ingestions.

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## Nonalcoholic fatty liver disease is associated with coronary artery disease in Koreans

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### Abstract

**AIM:** To investigate whether nonalcoholic fatty liver disease (NAFLD) affects coronary artery disease (CAD) and identify candidate mediators.

**METHODS:** Patients who underwent coronary angiography were consecutively recruited. The patients were classified into four groups by coronary artery stenosis: A, insignificant; B, one-vessel disease; C, two-vessel disease; and D, three-vessel disease. Abdominal ultrasonography was performed to determine the presence of a fatty liver and categorize by grade: 0, no evidence; 1, mild; 2, moderate; and 3, severe. We measured not only known CAD risk factors, but also serum insulin, HOMA-index, adiponectin, interleukin-6, tumor necrosis factor- $\alpha$  and high-sensitivity C-reactive protein levels.

**RESULTS:** Of the 134 patients who met the inclusion criteria, 82 (61.2%) had ultrasonographically diagnosed NAFLD. Among the 46 patients with CAD, 37 (80.4%) had evidence of a fatty liver. The two groups (A vs B-D) were significantly different in terms of age, total cholesterol, triglycerides, low-density lipoprotein levels and fatty liver. Coronary artery stenosis was strongly associated with fatty liver in a grade-dependent manner ( $P = 0.025$ ). In binary logistic regression, NAFLD was a significant independent predictor of CAD ( $P = 0.03$ , OR = 1.685; 95%CI: 1.051-2.702). Among the candidate mediators, the serum adiponectin level showed a trend toward lowering based on CAD progression ( $P = 0.071$ ).

**CONCLUSION:** NAFLD is an independent risk factor for CAD in a grade-dependent manner. Moreover, adiponectin might be related to the pathogenesis of NAFLD.

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**Key words:** Nonalcoholic fatty liver disease; Coronary artery disease; Coronary angiography; Adiponectin; Insulin resistance

**Core tip:** This article shows that angiographically proven coronary artery stenosis is strongly associated with nonalcoholic fatty liver disease (NAFLD) in a grade-dependent manner. Although many recent studies used coronary artery calcification score, carotid artery intima-media thickness, or carotid artery plaque measurements as surrogate markers for coronary artery disease (CAD), we evaluated the interaction between fatty liver and cardiovascular outcomes using coronary angiograms in a prospective case-controlled study. Because the pathogenesis of NAFLD and CAD is not fully elucidated, we attempted to identify mediators of these diseases and believe that adiponectin might be related to the development and progression of CAD in patients with NAFLD.

Choi DH, Lee SJ, Kang CD, Park MO, Choi DW, Kim TS, Lee W, Cho BR, Kim YH, Lee B, Ryu DR, Lee JW. Nonalcoholic fatty liver disease is associated with coronary artery disease in Koreans. *World J Gastroenterol* 2013; 19(38): 6453-6457 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6453.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6453>

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common disorder with an increasing prevalence of approximately 34% of the adult population in the United States<sup>[1]</sup>. Patients with NAFLD can progress to more aggressive forms of nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis, end-stage liver disease, and eventually hepatocellular carcinoma<sup>[2]</sup>. Because NAFLD is related to metabolic syndrome and obesity, many patients with NAFLD have coronary artery disease (CAD). Several studies have reported that NAFLD is a strong independent risk factor for CAD<sup>[3,4]</sup>. However, these studies have some clinical application limitations because of the use of indirect modalities, such as coronary artery calcification or intima-media thickness despite coronary artery imaging. Authors of these studies suggested that the presence of CAD was indicated by coronary artery calcification or intima-media thickness despite conducting coronary artery imaging<sup>[5,6]</sup>. Many NAFLD studies conducted in Western populations have found a relationship between NAFLD and CAD in relatively obese patients, which has not been found in Asian populations<sup>[6,7]</sup>. Therefore, the relationship between NAFLD and CAD in relatively thin Asian people must be evaluated. This study was conducted to evaluate whether NAFLD independently affects angiographically proven CAD in Asians and, if so, which mediator is responsible for this association.

## MATERIALS AND METHODS

### Subjects and study design

From January 2009 to June 27, 2011, 184 adult patients who underwent elective coronary angiography (CAG) at Kangwon National University Hospital were consecutively recruited. Indications for CAG included Canadian Cardiovascular Society class III or IV angina upon medical treatment, high-risk findings upon noninvasive testing, acute coronary syndrome, or a chest pain evaluation according to the American College of Cardiology/American Heart Association recommendations<sup>[8]</sup>. Standard selective CAG was performed by three experienced cardiologists and reviewed by another cardiologist. CAD was defined as the presence of at least a 50% stenosis in at least one major coronary artery. The patients were classified into four groups according to the number of major coronary arteries affected by CAD: A, insignificant coronary artery stenosis; B, one-vessel disease; C, two-vessel disease; and D, three-vessel disease.

We excluded patients with viral hepatitis (positive for

hepatitis B surface antigen and anti-hepatitis C virus), history of alcohol ingestion (> 30 g/d for men and > 20 g/d for women), history of drug use reported to cause steatosis (steroids, estrogens, tamoxifen, amiodarone, valproic acid, diltiazem, or methotrexate), improved steatosis (metformin, statins, or glitazones) within 3 mo of enrollment, or other history of chronic liver disease. Hypertension was defined as systolic blood pressure  $\geq$  140 mmHg, diastolic blood pressure  $\geq$  90 mmHg or using antihypertensive medications. Diabetes was defined as fasting blood sugar  $\geq$  126 mg/dL or using glucose-lowering medications (oral agents or insulin). Of the 184 patients, we excluded 50 with at least one potential cause for chronic liver disease. Altogether, 134 patients were enrolled and underwent abdominal ultrasonography within 2 d after CAG by a single experienced physician to determine the presence of four fatty liver grades: 0, no evidence of fatty liver; 1, mild; 2, moderate; and 3, severe degree. The presence of a fatty liver was identified by characteristic echo patterns such as a diffuse increase in the echogenicity of the liver compared with that of the kidney according to conventional criteria<sup>[9]</sup>. We measured not only known risk factors (*i.e.*, age, male gender, high low-density lipoprotein, low high-density lipoprotein, triglyceride, body mass index, diabetes, and hypertension) for CAD but also serum insulin, HOMA index, adiponectin, interleukin (IL)-6, tumor necrosis factor (TNF)- $\alpha$  and high-sensitivity C-reactive protein (hs-CRP) levels. This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Kangwon National University Hospital.

### Statistical analysis

Clinical and biochemical variables were compared between the two groups (A vs B-D). Continuous variables were assessed with the unpaired Student's *t*-test, and nominal variables were compared with the chi-square test. Variables that were significantly different between the two groups were extracted and included as covariates in a binary logistic regression with CAD as the dependent and NAFLD as the independent variable. Correlations between CAD severity and NAFLD degree were analyzed using Pearson's correlation analysis. A *P*-value < 0.05 was considered significant. All analyses were conducted using the SPSS for Windows 12.0.1 statistical software (SPSS, Inc., Chicago, IL, United States).

## RESULTS

A total of 134 (37 males and 97 females) patients met the inclusion criteria for the study. Table 1 demonstrates the demographic, clinical and laboratory data of the subjects without CAD (A) and those with CAD (B-D). The two groups were significantly different in terms of age, total cholesterol, triglycerides, low-density lipoprotein levels and presence of NAFLD. In addition, there tended to be more clinical features associated with metabolic syndrome in the CAD group, but the difference was not significant. In each

**Table 1** Comparison of clinical characteristics and laboratory data between subjects with and without coronary artery disease

	Group A Insignificant stenosis (n = 88)	Group B-D Significant stenosis (n = 46)	P value
Age (yr)	62.5 ± 10.8	65.2 ± 9.2	0.010
Sex (male)	20 (22.7)	17 (37.0)	0.104
DM	11 (12.5)	10 (21.7)	0.211
HTN	49 (55.7)	33 (71.7)	0.093
Height (cm)	155.0 ± 7.4	156.4 ± 8.4	0.333
Weight (kg)	61.9 ± 8.2	62.5 ± 10.0	0.734
BMI (kg/m <sup>2</sup> )	25.8 ± 3.3	25.6 ± 3.4	0.697
Waist circumference (cm)	86.8 ± 13.4	89.7 ± 6.9	0.169
Hip circumference (cm)	97.6 ± 13.8	98.7 ± 8.3	0.607
WHR	0.89 ± 0.9	0.91 ± 0.8	0.238
Total cholesterol (mg/dL)	177.1 ± 30.8	195.6 ± 39.1	0.009
HDL cholesterol (mg/dL)	41.2 ± 12.2	38.4 ± 12.1	0.227
Triglyceride (mg/dL)	134.9 ± 72.4	177.4 ± 94.4	0.012
Measured-LDL cholesterol (mg/dL)	102.3 ± 26.1	115.5 ± 33.3	0.015
Calculated-LDL cholesterol (mg/dL)	108.6 ± 28.3	121.7 ± 33.7	0.033
Creatinine (mg/dL)	0.8 ± 0.3	1.1 ± 0.4	0.068
Uric acid (mg/dL)	4.6 ± 1.4	4.9 ± 1.6	0.399
Hemoglobin (g/dL)	13.4 ± 1.8	13.0 ± 1.5	0.356
HbA1c (%)	5.7 ± 0.7	6.3 ± 1.2	0.072
Systolic BP (mmHg)	123.3 ± 16.6	125.6 ± 15.6	0.409
Diastolic BP (mmHg)	73.8 ± 10.7	75.9 ± 9.6	0.250
FBS (mg/dL)	104.2 ± 21.2	115.3 ± 37.3	0.082
Total bilirubin (mg/dL)	1.0 ± 0.5	1.1 ± 0.6	0.432
Albumin (g/dL)	3.9 ± 0.3	3.9 ± 0.4	0.465
AST (U/L)	34.1 ± 55.0	27.1 ± 11.0	0.394
ALT (U/L)	30.7 ± 53.4	22.8 ± 9.7	0.321
PT INR	0.9 ± 0.3	0.8 ± 0.1	0.182
HOMA-index	6.29 ± 9.16	5.99 ± 5.39	0.838
NAFLD	44 (51.2)	36 (78.3)	0.002

Data are expressed as mean ± SD or n (%). DM: Diabetes mellitus; HTN: Hypertension; BMI: Body mass index; WHR: Waist-hip ratio; HDL: High density lipoprotein; LDL: Low density lipoprotein; BP: Blood pressure; FBS: Fasting blood sugar; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PT: Prothrombin time; HOMA-index: Homeostatic model assessment of insulin resistance, fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5; NAFLD: Nonalcoholic fatty liver disease.

group, women were predominant, and all subjects were post-menopausal except for one person in the CAD group.

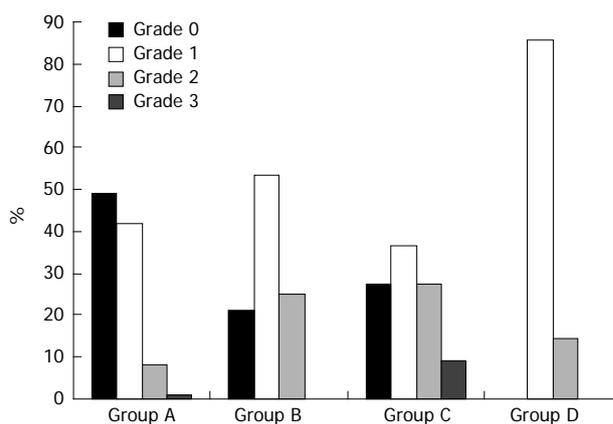
An analysis of the relationship between NAFLD and the presence of CAD is shown in Table 2. In addition to the significantly different variables between the two groups in Table 1, well-known established risk factors for CAD, such as age, gender, glucose, HbA1c and body mass index, were considered as covariates in conducting the multivariate analysis. In those models, as shown in Table 2, NAFLD was the significant independent predictor for CAD ( $P = 0.03$ , OR = 1.685; 95%CI: 1.051-2.702).

Next, we evaluated the correlation between the NAFLD degree and CAD severity. The proportion of patients with NAFLD increased from 51.1% in group A to 100% in group D. In group A, most of the fatty livers were grade 1. However, in the higher grade CAD group, the proportion of patients with more severe fatty livers was increased. No subject in group D (three-vessel

**Table 2** Multivariate analysis of coronary artery disease with age, nonalcoholic fatty liver disease and metabolic risk factors

	OR (95%CI)	P value
NAFLD	1.685 (1.051-2.702)	0.030
Age	1.056 (1.010-1.104)	0.057
Total cholesterol (mg/dL)	1.012 (0.982-1.043)	0.427
TG (mg/dL)	1.004 (0.998-1.010)	0.873
Measured-LDL cholesterol (mg/dL)	1.003 (0.970-1.036)	0.225

NAFLD: Nonalcoholic fatty liver disease; TG: Triglycerides; LDL: Low density lipoprotein.



**Figure 1** Relationship between the grade of nonalcoholic fatty liver disease and severity of coronary artery disease.

disease) had a normal liver. Figure 1 shows that angiographically proven coronary artery stenosis was strongly associated with NAFLD in a grade-dependent manner by Pearson's correlation analysis ( $P = 0.002$ ).

In addition, we measured the serum level of candidate mediators of metabolic syndrome, such as insulin, the HOMA index, IL-6, TNF- $\alpha$ , and hs-CRP (Table 3). In our results, none of the factors assessed were found to be related to CAD. However, serum adiponectin level demonstrated a trend toward lowering based on CAD progression ( $P = 0.071$ ).

## DISCUSSION

Our findings demonstrate that NAFLD is strongly associated with coronary artery stenosis in a grade-dependent manner. Our results also demonstrate that NAFLD is a significant predictor of CAD independent of traditional risk factors in Asians. Furthermore, we suggest that adiponectin might have a potential pathogenic role in the development and progression of CAD in patients with NAFLD.

Because NAFLD is a hepatic manifestation of metabolic syndrome, many studies have suggested that NAFLD results in increased cardiovascular risk and mortality<sup>[7,10]</sup>. The risk for developing cardiovascular morbidity and mortality is thought to be higher than the risk for developing hepatic disease because of its slow progression. Therefore, many studies have investigated the association between NAFLD and cardiovascular diseases. As a result,

**Table 3 Comparison of candidate mediators between subjects with and without coronary artery disease (mean  $\pm$  SD)**

	Group A Insignificant stenosis (n = 88)	Group B-D Significant stenosis (n = 46)	P value
Adiponectin ( $\mu\text{g}/\text{mL}$ )	8.40 $\pm$ 5.97	6.95 $\pm$ 5.85	0.071
IL-6 (pg/mL)	4.55 $\pm$ 7.75	4.71 $\pm$ 7.41	0.894
TNF- $\alpha$ (ng/mL)	4.00 $\pm$ 3.95	4.85 $\pm$ 4.73	0.273
hs-CRP(mg/dL)	0.45 $\pm$ 1.70	0.74 $\pm$ 1.18	0.366

IL: Interleukin; TNF: Tumor necrosis factor; hs-CRP: High sensitive C-reactive protein.

a number of studies have demonstrated that NAFLD is an independent risk factor for CAD<sup>[4,5,11-13]</sup>. However, most studies used coronary artery calcification score, carotid artery intima-media thickness, carotid artery plaque measurements, or circulatory endothelial dysfunction as surrogate markers for CAD<sup>[5,6,14]</sup>. Despite the fact that the coronary calcification score is a well-known marker for an increased risk of coronary events, the direct relationship between the presence of NAFLD and clinical CAD must be evaluated for use in the clinical setting<sup>[3]</sup>. Recently, Wong *et al*<sup>[15]</sup> evaluated the interaction between fatty liver and cardiovascular outcomes using coronary angiograms in a prospective cohort study and demonstrated that fatty liver is associated with CAD independently of other metabolic factors, which is consistent with our results. In contrast, our study was different from that study because we demonstrated that angiographically proven coronary artery stenosis was strongly associated with fatty liver in a grade-dependent manner.

Although the pathogenesis of NAFLD and CAD has not been fully elucidated, several explanations are present for the relationship between NAFLD and CAD. One widely accepted hypothesis implicates low-grade inflammatory conditions as key factors leading to hepatic steatosis and atherosclerosis<sup>[16,17]</sup>. Moreover, oxidative stress is presumed to play a role in NASH pathogenesis. Many investigators have studied additional mechanisms that might be associated with NAFLD, which are supported by the levels of various biomarkers, such as reactive oxygen species, adipocytokines (leptin and adiponectin), CRP, and caspase-generated cytokeratin-18<sup>[18-21]</sup>. In this study, we also tried to find candidate mediators of the mechanism of this relationship. We investigated several mediators, including adiponectin, IL-6, TNF- $\alpha$ , and hs-CRP. Among these candidate mediators, adiponectin may have been related to the development and progression of CAD in patients with NAFLD in our study. Adiponectin is the most abundant adipose-specific adipokine, and decreases hepatic insulin resistance and attenuates liver inflammation<sup>[22]</sup>. Low levels of serum adiponectin might play an important role in the pathogenesis of clinical CAD and NAFLD. In contrast, NAFLD is also characterized by increased insulin resistance<sup>[23]</sup>. We measured fasting serum insulin levels and calculated the HOMA index to confirm this relationship in our study. Because we included obese Asians, which in contrast with previous

Asian-Pacific NAFLD studies that included non-obese subjects, our study subjects had relatively high insulin resistance<sup>[24]</sup>. However, fasting serum insulin levels and HOMA-IR were not different between our two groups (with/-without CAD and with/-without a fatty liver).

Some limitations of our study merit comment. First, our results were not based on a biopsy-proven NAFLD. There is no histology or staging of fibrosis by use of elastography to determine the liver fibrosis. We diagnosed NAFLD using hepatic ultrasonography. This technique does not identify fatty infiltration < 30% although it is a safe and confirmed reliable noninvasive method<sup>[25]</sup>. This technique also has additional weak points, which are intra- and interobserver differences when making a diagnosis. However, to overcome these limitations, ultrasonography was performed by a single experienced physician to determine the presence of the four fatty liver grades. In addition, standard selective CAG was performed to diagnose and measure CAD severity by three experienced cardiologists in our study. To reduce interobserver variability for CAG, another cardiologist also reviewed all of the data. Second, this study was conducted at a single center in a rural area, which increased the chance for selection bias. Women were predominant in the included subject. A possible explanation for this gender imbalance is that men in this area had a high prevalence of alcohol intake and were excluded based on a history of alcohol ingestion.

Because NAFLD is considered a hepatic manifestation of metabolic syndrome, many studies have investigated the association between NAFLD and cardiovascular diseases. As a result, our study demonstrates that NAFLD is an independent risk factor for angiographically proven CAD in a grade-dependent manner. Because the pathogenesis of NAFLD and CAD are not fully elucidated, we also attempted to identify mediators and believe that adiponectin might be related to the development and progression of CAD in patients with NAFLD. Therefore, future large-scale studies are needed to elucidate the precise mechanism of this relationship.

## COMMENTS

### Background

Although recent many studies used coronary artery calcification score, carotid artery intima-media thickness, or carotid artery plaque measurements as surrogate markers for coronary artery disease (CAD), this study evaluated the interaction between fatty liver and cardiovascular outcomes using coronary angiograms in a prospective case-control study of Asians.

### Research frontiers

The relationship between nonalcoholic fatty liver disease (NAFLD) and CAD in relatively thin Asian people must be evaluated. Moreover, because the pathogenesis of NAFLD and CAD are not fully elucidated, the authors attempted to identify candidate mediators.

### Innovations and breakthroughs

This article show that angiographically proven coronary artery stenosis was strongly associated with NAFLD in a grade-dependent manner. In addition, the authors attempted to identify mediators and believe that adiponectin might be related to the development and progression of CAD in patients with NAFLD.

### Applications

By understanding the association between NAFLD and CAD, patients with a se-

vere degree of fatty liver disease have to be concerned about CAD to improve their prognosis.

### Peer review

This is a prospective single center study, which investigate the relationship between NAFLD and CAD and seeks candidate mediators. Future large-scale studies are needed to elucidate the precise mechanism of this relationship.

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## Diet of patients after pouch surgery may affect pouch inflammation

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### Abstract

**AIM:** To investigate the diet of pouch patients compared to healthy controls, and to correlate pouch patients' diet with disease behavior.

**METHODS:** Pouch patients were recruited and prospectively followed-up at the Comprehensive Pouch Clinic at the Tel Aviv Sourasky Medical Center. Pouch behavior was determined based on clinical, endoscopic and histological criteria. Healthy age- and sex-matched volunteers were selected from the "MABAT" Israeli

Nutrition and Public Health Governmental Study and served as the control group. All the participants completed a 106-item food frequency questionnaire categorized into food groups and nutritional values based on those used in the United States Department of Agriculture food pyramid and the Israeli food pyramid. Data on Dietary behavior, food avoidance, the use of nutritional supplements, physical activity, smoking habits, and body-mass index (BMI) were also obtained. Pouch patients who had familial adenomatous polyposis ( $n = 3$ ), irritable pouch syndrome ( $n = 4$ ), or patients whose pouch surgery took place less than one year previously ( $n = 5$ ) were excluded from analysis.

**RESULTS:** The pouch patients ( $n = 80$ ) consumed significantly more from the bakery products food group ( $1.2 \pm 1.4$  servings/d vs  $0.6 \pm 1.1$  servings/d,  $P < 0.05$ ) and as twice as many servings from the oils and fats ( $4.8 \pm 3.4$  servings/d vs  $2.4 \pm 2$  servings/d,  $P < 0.05$ ), and the nuts and seeds food group ( $0.3 \pm 0.6$  servings/d vs  $0.1 \pm 0.4$  servings/d,  $P < 0.05$ ) compared to the controls ( $n = 80$ ). The pouch patients consumed significantly more total fat ( $97.6 \pm 40.5$  g/d vs  $84.4 \pm 39$  g/d,  $P < 0.05$ ) and fat components [monounsaturated fatty acids ( $38.4 \pm 16.4$  g/d vs  $30 \pm 14$  g/d,  $P < 0.001$ ), and saturated fatty acids ( $30 \pm 15.5$  g/d vs  $28 \pm 14.1$  g/d,  $P < 0.00$ )] than the controls. In contrast, the pouch patients consumed significantly fewer carbohydrates ( $305.5 \pm 141.4$  g/d vs  $369 \pm 215.2$  g/d,  $P = 0.03$ ), sugars ( $124 \pm 76.2$  g/d vs  $157.5 \pm 90.4$  g/d,  $P = 0.01$ ), theobromine ( $77.8 \pm 100$  mg/d vs  $236.6 \pm 244.5$  mg/d,  $P < 0.00$ ), retinol ( $474.4 \pm 337.1$   $\mu$ g/d vs  $832.4 \pm 609.6$   $\mu$ g/d,  $P < 0.001$ ) and dietary fibers ( $26.2 \pm 15.4$  g/d vs  $30.7 \pm 14$  g/d,  $P = 0.05$ ) than the controls. Comparisons of the food consumption of the patients without ( $n = 23$ ) and with pouchitis ( $n = 45$ ) showed that the former consumed twice as many fruit servings as the latter ( $3.6 \pm 4.1$  servings/d vs  $1.8 \pm 1.7$  servings/d, respectively,  $P < 0.05$ ). In addition, the pouchitis patients consumed significantly fewer liposoluble antioxidants, such as cryptoxanthin ( $399 \pm 485$

$\mu\text{g/d}$  vs  $890.1 \pm 1296.8 \mu\text{g/d}$ ,  $P < 0.05$ ) and lycopene ( $6533.1 \pm 6065.7 \mu\text{g/d}$  vs  $10725.7 \pm 10065.9 \mu\text{g/d}$ ,  $P < 0.05$ ), and less vitamin A ( $893.3 \pm 516 \mu\text{g/d}$  vs  $1237.5 \pm 728 \mu\text{g/d}$ ,  $P < 0.05$ ) and vitamin C ( $153.3 \pm 130 \text{ mg/d}$  vs  $285.3 \pm 326.3 \text{ mg/d}$ ,  $P < 0.05$ ) than the patients without pouchitis. The mean BMI of the pouchitis patients was significantly lower than the BMI of the patients with a normal pouch:  $22.6 \pm 3.2$  vs  $27 \pm 4.9$  ( $P < 0.001$ ).

**CONCLUSION:** Decreased consumption of antioxidants by patients with pouchitis may expose them to the effects of inflammatory and oxidative stress and contribute to the development of pouchitis.

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**Key words:** Ulcerative colitis; Dietary reference intake; Body mass index; Ileal-pouch anal anastomosis; Pouch surgery; Food frequency questionnaire

**Core tip:** The diet of patients who had pouch surgery differed significantly from that of healthy individuals. Patients with pouchitis consumed significantly fewer fruit servings and antioxidants than patients with normal pouches, thus possibly exposing the former to inflammatory and oxidative stress. The body mass index of patients with pouchitis was significantly lower than patients with normal pouches, probably as a result of the continuous inflammatory burden.

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## INTRODUCTION

Total proctocolectomy and the formation of a small bowel reservoir-ileal pouch anal anastomosis (IPAA, “pouch surgery”) is the surgery of choice for the treatment of severe, refractory or complicated ulcerative colitis (UC)<sup>[1,2]</sup>. Pouch surgery has good short- and long-term outcomes and is associated with improved quality of life<sup>[2]</sup>. Inflammation of the pouch (“pouchitis”) is the most common long-term complication, with a reported incidence of up to 60%<sup>[3]</sup>. Nutrition is increasingly incorporated into the management of inflammatory bowel disease (IBD)<sup>[4]</sup>. However, few studies have assessed the influence of nutrition on the health status of pouch patients. Rather most have focused on patients’ subjective feelings after consuming specific food products<sup>[5]</sup>. Nevertheless, the potential contribution of nutrition to the development of inflammation in the pouch, as well as to IBD in general, remains unclear. Studies have shown that probiotic supplements, such as various strains of lactobacilli 3

(VSL#3), may prevent pouchitis after closure of the ileostomy, shorten the duration of the inflammation, and maintain remission<sup>[6,7]</sup>. Several nutritional imbalances may also result from pouch surgery itself, including vitamin B12 and iron deficiency, fat malabsorption and electrolyte and trace element deficiencies<sup>[8-11]</sup>. Such deficiencies may, in turn, cause or increase inflammation by mechanisms such as increased tissue oxidative stress<sup>[10]</sup>.

We hypothesized that diet modification and nutritional imbalance may occur after pouch surgery and that these could be associated with and predispose pouch patients to the development of pouchitis. We further assumed that such major changes might be related to the consumption of essential vitamins, minerals, antioxidants or fibers, which could thus potentially contribute to pouch inflammation<sup>[11-15]</sup>. The purpose of the current investigation was thus to gather and analyze the detailed intake of food groups and nutrients as well as examine the nutritional and lifestyle habits of pouch patients, and test for correlations between these parameters and the occurrence of pouchitis.

## MATERIALS AND METHODS

### Study population

Pouch patients were recruited from the Comprehensive Pouch Clinic at the Tel Aviv Sourasky Medical Center (Tel Aviv, Israel), a tertiary referral center for IBD and the national referral center for pouch patients. Both an IBD-oriented gastroenterologist (Dotan I) and a colorectal surgeon (Tulchinsky H) examined all pouch patients. Healthy age- and sex-matched volunteers were selected from the “MABAT” Israeli Nutrition and Public Health Governmental Study cohort<sup>[16]</sup>. Pouchitis was diagnosed by accepted clinical, endoscopic and histological criteria (the pouchitis disease activity index, PDAI<sup>[17]</sup>). Pouch status was further defined as normal or pouchitis (recurrent acute pouchitis and chronic pouchitis) as previously described<sup>[2]</sup>. Briefly, normal pouch status was defined as no clinical, endoscopic or histological criteria for pouchitis during the previous 2 years and no antibiotic or anti-inflammatory therapy of any type. Chronic pouchitis was defined by clinical, endoscopic and histological criteria that called for chronic administration of antibiotics or anti-inflammatory therapies for more than one month or when there were more than 5 flares of pouchitis within a year<sup>[2,18,19]</sup>. Recurrent acute pouchitis was defined as  $\leq 5$  flares of pouchitis responding to a 2-wk course of antibiotics/year. The data on pouch patients who had familial adenomatous polyposis ( $n = 3$ ), irritable pouch syndrome ( $n = 4$ ), or patients who had had their pouch for less than one year ( $n = 5$ ) were excluded.

Since there was no significant difference in the food and nutrient consumption between the patients with chronic pouchitis and those with recurrent acute pouchitis, they were combined into a single “pouchitis” group. The data for all of the enrolled pouch patients were compared to those of the healthy controls. Patients with a normal pouch status ( $n = 23$ ) were further compared to

**Table 1 Food group consumption in pouch patients and healthy controls (mean  $\pm$  SD)**

Food group	Consumption healthy controls ( <i>n</i> = 80, servings/d)	Consumption pouch patients ( <i>n</i> = 80, servings/d)	Recommendation <sup>1</sup>	<i>P</i> value
Grains	6.1 $\pm$ 4.0	6.9 $\pm$ 4.01	6-11 <sup>2</sup>	0.213
Bakery	0.6 $\pm$ 1.1	1.2 $\pm$ 1.4	6-11 <sup>2</sup>	0.030
Potatoes	0.5 $\pm$ 0.5	0.7 $\pm$ 0.6	6-11 <sup>2</sup>	0.063
Vegetables	3.9 $\pm$ 2.7	3.6 $\pm$ 2.7	3-5	0.49
Fruits	2.2 $\pm$ 2.1	2.5 $\pm$ 2.8	2-4	0.47
Dairy	3.7 $\pm$ 2.6	4 $\pm$ 3.3	2-3	0.52
Meat, fish and poultry	2.4 $\pm$ 2.2	2.3 $\pm$ 1.6	2-3 <sup>3</sup>	0.945
Eggs	0.4 $\pm$ 0.5	0.5 $\pm$ 0.4	2-3 <sup>3</sup>	0.206
Legumes	0.4 $\pm$ 0.6	0.3 $\pm$ 0.5	2-3 <sup>3</sup>	0.094
Oils and fats	2.4 $\pm$ 2.0	4.8 $\pm$ 3.4	Limited	0.000
Nuts and seeds	0.1 $\pm$ 0.4	0.3 $\pm$ 0.6	Limited	0.012
Snacks and soft drinks	4.5 $\pm$ 3.0	5.0 $\pm$ 4.5	Limited	0.353
Water	6.0 $\pm$ 3.9	6.0 $\pm$ 3.5	-	0.913

<sup>1</sup>Serving recommendations according to food pyramid (*n* = 20); <sup>2</sup>6-11 servings are recommended for the grains, baked goods or potato categories; <sup>3</sup>2-3 servings are recommended for the meat, fish and poultry, eggs, and legume categories.

patients with pouchitis (*n* = 45). All participants gave their informed consent. The study complied with the Helsinki Declaration and the ethical guidelines of our institution.

### Questionnaires

All participants were prospectively interviewed using a food frequency questionnaire (FFQ). The 106 items on the FFQ were categorized into food groups according to those defined in the United States Department of Agriculture (USDA) food pyramid<sup>[20]</sup> and the Israeli food pyramid<sup>[21]</sup>. The questionnaire also included sub-food groups defined in the "MABAT" Israeli Nutrition and Public Health Governmental Study<sup>[16]</sup>. The nutritional values of the food items were taken from the USDA FNDD, version 4.1<sup>[22]</sup>. The nutritional values of several specific Israeli food items that do not appear in the USDA FNDD database were taken from the Israeli Ministry of Health food consumption and nutrients "TZAMERET" database, version 2<sup>[23]</sup>. Pouch patients were also asked about their dietary behavior, food avoidance, and the use of nutritional supplements, as well as physical activity, smoking habits, and body-mass index (BMI). Assessment of the questionnaires was based on the recommended range of values established by the USDA FNDD<sup>[22]</sup> and Israeli Health Ministry "TZAMERET"<sup>[23]</sup> databases. The nutrient consumption of all participants was compared to the upper limits for daily nutrient recommendations for healthy males and females between the ages of 31-50 years as indicated in the USDA Dietary Reference Intake (DRI) 2010<sup>[24]</sup>.

### Statistical analysis

Statistical analyses were conducted using SPSS software version 19.0 (SPSS Inc. Headquarters, S Wacker Drive, Chicago, IL, United States). A *P* value of < 0.05 was considered significant. Data are presented as mean  $\pm$  SD for continuous variables, and frequencies and percentages for categorical variables. Independent *t*-tests were used to compare pouch patients *vs* healthy controls for

food group and nutrient consumption. Fisher's exact test and independent *t*-tests were used to compare normal pouch patients to recurrent acute and chronic pouchitis patients for the categorical and continuous variables, respectively.

## RESULTS

### Diets of pouch patients vs controls

Eighty adult pouch patients were recruited and compared to 80 healthy adult volunteers. Subjects from both groups were matched for sex and age. Differences in their nutritional intake were first examined by comparing their consumption of servings of the main food groups. The major food groups were divided into subgroups based on the "MABAT" study distribution<sup>[16]</sup>. The pouch patients consumed significantly more bakery, oils and fats, and nuts and seeds compared to the controls (Table 1). The consumption of other food groups was comparable. The total nutrient content of foods<sup>[25]</sup> consumed by the patients and the control groups is shown in Table 2. The pouch patients' increased consumption of fat servings included significantly more total fat and fat components; *i.e.*, mono-unsaturated fatty acids and saturated fatty acids, than the controls. The pouch patients also consumed significantly higher amounts of several nutrients than the controls, *e.g.*, niacin, zinc, and vitamins C and D (Table 2). These higher levels were usually attributed to external supplements rather than to the diet itself. In contrast, the pouch patients consumed significantly fewer carbohydrates, sugars, theobromine, retinol and dietary fibers compared to the controls. Interestingly, neither the controls nor the pouch patients met DRI recommendations for dietary fiber intake (38 g/d<sup>[24]</sup>).

### Normal pouch vs pouchitis patients' diets

The pouch patients were divided into a normal pouch group (*n* = 23) and a pouchitis group (*n* = 45) (both recurrent acute and chronic). The demographic parameters of

**Table 2** Consumption of nutrients in pouch patients and healthy controls (mean  $\pm$  SD)

Nutrient	Consumption healthy controls ( <i>n</i> = 80)	%DRI	Consumption pouch patients ( <i>n</i> = 80)	%DRI	<i>P</i> value
Energy (kcal)	2655.2 $\pm$ 1313.7	-	2509.9 $\pm$ 986.4	-	0.430
Proteins (g)	112.8 $\pm$ 59.4	200%	113 $\pm$ 42.5	201%	0.977
Total fat (g)	84.4 $\pm$ 39	-	97.6 $\pm$ 40.5	-	0.038
Carbohydrates (g)	369.0 $\pm$ 215.2	284%	305.5 $\pm$ 141.4	234%	0.029
Theobromine (mg)	236.6 $\pm$ 244.5	-	77.8 $\pm$ 100	-	< 0.001
Total sugars (g)	157.5 $\pm$ 90.4	-	124 $\pm$ 76.2	-	0.012
Total dietary fiber (g)	30.7 $\pm$ 14.0	80%	26.2 $\pm$ 15.4	69%	0.055
Zinc (mg)	13.3 $\pm$ 6.5	121%	16.6 $\pm$ 9.2	151%	0.01
Retinol ( $\mu$ g)	832.4 $\pm$ 609.6	-	474.4 $\pm$ 337.1	-	< 0.001
Vitamin D ( $\mu$ g)	7.9 $\pm$ 5.7	158%	15.7 $\pm$ 19.9	314%	0.001
Vitamin C (mg)	148.2 $\pm$ 80.6	164%	210.3 $\pm$ 225.4	233%	0.022
Niacin (mg)	34.4 $\pm$ 20.0	212%	43.0 $\pm$ 18.1	269%	0.005
Total monounsaturated fatty acids (g)	30 $\pm$ 14	-	38.4 $\pm$ 16.4	-	< 0.001
Total polyunsaturated fatty acids (g)	17.9 $\pm$ 9.2	-	20.4 $\pm$ 9.2	-	0.082
Total saturated fatty acids (g)	28.0 $\pm$ 14.1	-	30 $\pm$ 15.5	-	0.006
Total W3 fatty acids (g)	0.14 $\pm$ 0.13	-	1.2 $\pm$ 3.4	-	0.03
Total W6 fatty acids (g)	16.8 $\pm$ 9.0	-	19.9 $\pm$ 9.1	-	0.4

DRI: Dietary reference intakes.

**Table 3** Demographic characteristics of pouch patient subgroups

	Normal pouch ( <i>n</i> = 23)	Recurrent acute and chronic pouchitis ( <i>n</i> = 45)	<i>P</i> value
Male/female	11/12	22/23	0.56
Age (yr)	53.2 $\pm$ 13.7	43.0 $\pm$ 14.9	< 0.001
Mean time since surgery (yr)	7.8 $\pm$ 4.4	11.0 $\pm$ 6.3	0.04
Operation stages (1/2/3)	4/16/3	4/34/7	0.4
Body mass index (kg/m <sup>2</sup> )	27 $\pm$ 4.9	22.6 $\pm$ 3.2	< 0.001
Food avoidance	60.90%	73.30%	0.21
Probiotics consumption	30.40%	31.10%	0.6
Vitamins/supplement consumption	43.50%	66.70%	0.06
Smokers	4.30%	13.30%	0.24

the two groups are shown in Table 3. Comparison of the food consumption of the normal pouch patients to that of patients with pouchitis revealed significant differences in two main food groups; namely, fruits and vegetables (Table 4). Patients with a normal pouch consumed twice as many fruit servings as patients with pouchitis ( $P < 0.01$ ) and tended to consume more vegetable servings than the pouchitis patients ( $P < 0.01$ ). The consumption of other food groups was comparable regardless of pouch status. We hypothesized that these findings would be reflected in significantly less consumption of antioxidants. As predicted, pouchitis patients consumed significantly less liposoluble antioxidants, such as cryptoxanthin and lycopene, as well as less vitamins A and C than the normal pouch patients. Taken together, these data suggest that patients with pouchitis may be more exposed to oxidative stress as a result of their consumption of fewer fruits and vegetables. Interestingly, two-thirds of the patients with pouchitis supplemented their diet with vitamins and minerals, compared to 43.5% of the patients with a normal pouch ( $P = 0.06$ ). Nevertheless, even after this supplementation, the total consumption of antioxidants was still significantly lower in the pouchitis group than in the normal pouch group. Seventy percent of all pouch

patients reported some type of food avoidance. The most frequently avoided foods were milk, citrus fruits, and spicy foods. Although up to 25% of all pouch patients avoided milk products, they met the recommended calcium intake level, mostly through supplements.

Only 26.2% ( $n = 21$ ) of all pouch patients in the cohort used probiotics; 30.4% ( $n = 7$ ) in the normal pouch group and 31.1% ( $n = 14$ ) in the pouchitis group. Most of these were over-the-counter probiotics, and only 4 patients used the probiotic VSL#3.

### BMI comparisons

Despite the comparable mean energy intake of patients with normal pouches and those with pouchitis, the mean BMI of both groups was significantly different, with the former having a significantly higher BMI than the latter. In terms of the normal BMI range for the healthy population (18.5-25 kg/m<sup>2</sup>)<sup>[26]</sup>, 15 patients (65%) in the normal pouch group fell into the overweight range compared to 35 patients (77%) the pouchitis group who were categorized in the normal or underweight range. This may suggest that inflammatory activity itself, rather than decreased caloric intake, plays a role in the significantly lower BMI of patients with pouchitis.

**Table 4 Food group consumption in patients with recurrent acute and chronic pouchitis *vs* patients with a normal pouch (mean  $\pm$  SD)**

Food group	Consumption normal pouch patients ( <i>n</i> = 23, servings/d)	Consumption recurrent acute and chronic pouchitis patients ( <i>n</i> = 45, servings/d)	Recommendation <sup>1</sup>	<i>P</i> value
Grains	7.0 $\pm$ 3.5	7.3 $\pm$ 4.5	6-11 <sup>2</sup>	0.7
Bakery	1.0 $\pm$ 1.4	1.2 $\pm$ 1.3	6-11 <sup>2</sup>	0.4
Potatoes	0.5 $\pm$ 0.4	0.8 $\pm$ 0.6	6-11 <sup>2</sup>	0.15
Vegetables	4.5 $\pm$ 3.0	3.3 $\pm$ 2.1	3-5	0.06
Fruits	3.6 $\pm$ 4.1	1.8 $\pm$ 1.7	2-4	0.015
Dairy	4.3 $\pm$ 3.0	3.7 $\pm$ 3.0	2-3	0.43
Meat, fish and poultry	2.4 $\pm$ 1.5	2.4 $\pm$ 1.8	2-3 <sup>3</sup>	0.99
Eggs	0.6 $\pm$ 0.5	0.5 $\pm$ 0.4	2-3 <sup>3</sup>	0.37
Legumes	0.2 $\pm$ 0.4	0.3 $\pm$ 0.5	2-3 <sup>3</sup>	0.28
Oils and fats	5.3 $\pm$ 3.0	4.7 $\pm$ 3.8	Limited	0.5
Nuts and seeds	0.4 $\pm$ 0.8	2.3 $\pm$ 0.4	Limited	0.55
Snacks and soft drinks	4.4 $\pm$ 3.0	5.5 $\pm$ 5.0	Limited	0.38
Water	6.0 $\pm$ 3.9	6.0 $\pm$ 3.5	-	0.93

<sup>1</sup>Serving recommendations according to food pyramid (*n* = 20); <sup>2</sup>6-11 servings are recommended for the grains, baked goods or potato categories; <sup>3</sup>2-3 servings are recommended for the meat, fish and poultry, eggs, and legume categories.

**Table 5 Consumption of nutrients in patients with recurrent acute and chronic pouchitis *vs* patients with a normal pouch (mean  $\pm$  SD)**

Nutrient	Consumption normal pouch patients ( <i>n</i> = 23)	%DRI	Consumption recurrent acute and chronic pouchitis patients ( <i>n</i> = 45)	%DRI	<i>P</i> value
Energy (kcal)	2592.7	-	2538.2	-	0.836
Proteins (g)	117.9	210%	113.1	201%	0.667
Total fat (g)	98.2	-	99.5	-	0.882
Carbohydrates (g)	321.3	247%	307.3	236%	0.709
Vitamin A-RAE ( $\mu$ g)	1237.5 $\pm$ 728.0	137%	893.3 $\pm$ 516.0	99%	0.027
Beta-carotene ( $\mu$ g)	7180.5 $\pm$ 7394.1	66%	4453 $\pm$ 4960.6	41%	0.075
Cryptoxanthin ( $\mu$ g)	890.1 $\pm$ 1296.8	-	399 $\pm$ 485	-	0.027
Lycopene ( $\mu$ g)	10725.7 $\pm$ 10065.9	-	6533.1 $\pm$ 6065.7	-	0.036
Vitamin C (mg)	285.3 $\pm$ 326.3	316%	153.35 $\pm$ 130	170%	0.02

RAE: Retinol activity equivalents; DRI: Dietary reference intake.

## DISCUSSION

Increased attention has been paid in recent years to the role of nutrition in the treatment of IBD patients<sup>[4,27]</sup>, and its putative contribution to inflammation continues to be a topic of considerable interest<sup>[14,15]</sup>. UC patients undergoing pouch surgery are exposed not only to the consequences of total removal of the large bowel and reconstruction of an ileal reservoir, but also to the potential influence of nutrition on inflammatory processes. Thus it is surprising that there are no nutritional guidelines for these patients. Moreover, there is only sparse information on nutrition among pouch patients and its relationships to the development, treatment, and prevention of pouch inflammation. In this prospective cross-sectional study, we employed the FFQ to characterize pouch patients' dietary consumption to analyze correlations between diet and pouch inflammation. We hypothesized that nutrition could be significantly impaired in these patients, which would have possible implications for the inflammation of the pouch.

The results indicate major differences in the diet of pouch patients as compared to healthy individuals and, more importantly, between patients with normal pouches and those with pouchitis. In particular, pouch patients

consumed significantly higher servings of fats and oils compared to healthy controls, and patients with pouchitis consumed fewer fruit servings and antioxidants than patients with a normal pouch. These findings on fat and oil consumption may be crucial since USDA nutritional guidelines recommend that fats should be consumed sparingly<sup>[20,28]</sup>. Sakamoto *et al.* for instance found that high consumption of fats and oils is associated with increased risk of CD<sup>[12]</sup>. The same may apply to the development of pouchitis, which, similar to CD, is an inflammation of the small bowel in an IBD patient.

Our patients with normal pouches consumed twice as many servings from the fruit food group than the pouchitis patients (Table 4). They also tended to consume more servings from the vegetable food group. Low consumption of fruits and vegetables has been shown to be inversely related to inflammation, as reflected by higher CRP levels<sup>[29]</sup>. El Muhtaseb *et al.*<sup>[10]</sup> for instance showed that pouch patients have significantly lower plasma concentrations of liposoluble antioxidants such as beta carotene, and that they have increased oxidative stress in plasma compared to healthy controls. This may imply that the low consumption of antioxidants and vitamin C observed in the pouchitis patients here may contribute to their low serum levels. According to D'Odorico *et al.*<sup>[30]</sup>

this may lead to further oxidative damage. When DRI consumption of dietary fibers is below the recommended level, several mechanisms may lead to a similar effect<sup>[31]</sup>. Intestinal bacteria ferment soluble fibers, producing short chain fatty acids such as butyrate<sup>[31,32]</sup> as well as lactic acid<sup>[32]</sup>. A shortage in butyrate was shown to be associated with the development of pouchitis<sup>[32]</sup>. Second, lactic acid decreases fecal pH<sup>[31]</sup>, which may contribute to protection from pouchitis<sup>[31]</sup> by inhibiting the proteolytic activity of bacterial glycosidases<sup>[33]</sup>.

Taken together, these results on the low consumption of antioxidants, vitamins and dietary fibers by pouchitis patients support our hypothesis that these imbalances may both predispose and be associated with the development of pouchitis in pouch patients. Whether the consumption of more antioxidants and vitamins can prevent further intestinal inflammation or even reverse it is an open question reserved for future studies. Notably, probiotic supplements were consumed by 26.2% of our pouch patients, but the probiotic formula VSL#3 that has been reported to be beneficial for the prevention of pouchitis<sup>[34]</sup> was consumed by only 5%. This low rate of use may change in the near future since 2011 VSL#3 has now been included in the Israeli MOH health basket as a supplement for patients with pouchitis<sup>[35]</sup>.

A major finding of the current work is the correlation between BMI and the inflammatory state. Patients with normal pouches had significantly higher BMI ratios than patients with pouchitis, even to the point of being in the “overweight” range<sup>[26]</sup>. This finding is intriguing given that there was no difference in energy intake between the normal pouch and the chronic pouchitis patient groups. Thus, differences in BMI might be due to increased malabsorption<sup>[3]</sup>, increased energy expenditure<sup>[3]</sup> or differences in microbiota composition, which may lead to differential utilization of nutrients<sup>[36]</sup>. This correlation between BMI and pouch inflammatory state also suggests that the inflammation itself contributes to energy expenditure, as we reported elsewhere for CD patients<sup>[3]</sup>.

In conclusion, the results of this study revealed significant differences in the consumption of food groups and nutrients between healthy controls and pouch patients, and between patients with normal pouches and those with pouchitis. These differences correlated, in part, with pouchitis and affected the patients’ BMI levels. Further studies on the mechanistic effects of nutrition on pouch inflammation are needed to help provide guidelines for nutritional counseling and interventions to alleviate the symptoms of pouchitis and modify its course.

## COMMENTS

### Background

Total proctocolectomy and the formation of a small bowel reservoir-ileal pouch anal anastomosis (IPAA, “pouch surgery”) is the surgery of choice for the treatment of severe, refractory or complicated ulcerative colitis (UC). Inflammation of the pouch (“pouchitis”) is the most common long-term complication, with a reported incidence of up to 60%. Nutrition has been increasingly incorporated into the management of inflammatory bowel disease (IBD). However, few studies have assessed the influence of nutrition on the health of pouch patients.

Moreover, the potential contribution of nutrition to the development of inflammation in the pouch, as well as in IBD in general, remains under-researched.

### Research frontiers

The characteristics of pouch patients’ nutrition were prospectively evaluated using a food frequency questionnaire. The questionnaire data were analyzed for correlations between pouch disease behavior, as determined by clinical, endoscopic and histological criteria. Most previous nutritional studies on pouch patients have focused on their subjective feelings after consuming specific food products rather than on the overall relationships between various food groups and nutrients and pouch disease behavior.

### Innovations and breakthroughs

The dietary intake and nutrient composition of pouch patients was analyzed for relationships with pouch disease behavior. The key finding shows that the diet of patients with pouch surgery differed significantly from that of healthy individuals. Moreover, patients with pouchitis consume significantly fewer fruit servings and antioxidants compared to patients with normal pouches, possibly exposing the former to inflammatory and oxidative stress. The body mass index of patients with pouchitis was significantly lower than patients with normal pouches, probably as a result of the continuous inflammatory burden.

### Applications

The findings suggest that the consumption of fruits and vegetables, as well as supplementation with specific vitamins, minerals and antioxidants may be beneficial for patients with pouchitis. Specific nutritional consultation for pouch patients is advisable.

### Terminology

Pouch surgery: This is the surgery of choice for the treatment of severe, refractory or complicated UC. The large bowel and the rectum are removed (total proctocolectomy), and a reservoir (“pouch”) constructed of the normal small bowel is created and connected to the anus (IPAA). Pouchitis: Inflammation of the small bowel (that was originally normal, not inflamed) creating the pouch.

### Peer review

This research compares the dietary and nutritional treatment of pouch patients and pouchitis. This manuscript is a meaningful and enlightening study in general because it reveals the dietary differences between pouch patients and controls as well as between patients with or without pouchitis, which can guide further investigations on this topic.

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## Consumption of spicy foods and the prevalence of irritable bowel syndrome

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### Abstract

**AIM:** To explore the association between consumption

of spicy foods and prevalence of irritable bowel syndrome (IBS) among Iranian adults.

**METHODS:** In this cross-sectional study, data from 4763 Iranian adult participants were used. Consumption of spicy foods was estimated using a dietary habits questionnaire that included a question on spicy foods consumption: "how frequently do you use spicy foods (pepper, curry, ginger, cinnamon and turmeric) during a week?" Participants could respond to the question by choosing one of these choices: never, 1-3 times, 4-6 times, 7-9 times, or more than 10 times per week. A modified Persian version of the Rome III questionnaire was used to determine the prevalence of IBS.

**RESULTS:** IBS was prevalent in 21.7% (18.6% of men and 24.1% of women) of the study population. After controlling for potential confounders including dietary behaviors, those consuming spicy foods  $\geq 10$  times per week were 92% more likely to have IBS compared with those who never consumed spicy foods (OR = 1.92; 95%CI: 1.23-3.01,  $P_{\text{trend}} < 0.01$ ). The association remained significant even after taking lactose intolerance into account (OR = 1.85; 95%CI: 1.18-2.90,  $P_{\text{trend}} < 0.01$ ). Stratified analysis by gender revealed that the association between consumption of spicy foods and IBS was not significant in men; however, a significant association was found among women after taking potential cofounders, including meal regularity and lactose intolerance, into account. Women who consumed spicy foods  $\geq 10$  times per week were two times more likely to have IBS compared with those who never consumed spicy foods (OR = 2.03; 95%CI: 1.09-3.77,  $P_{\text{trend}} = 0.02$ ).

**CONCLUSION:** Consumption of spicy foods is directly associated with IBS, particularly in women. Further, prospective studies are warranted to (1) examine this association in other populations; and (2) evaluate whether dietary interventions, for example a reduction

in spice consumption, would improve IBS symptoms.

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**Key words:** Spice; Diet; Condiments; Red pepper; Irritable bowel syndrome; Functional gastrointestinal disorders

**Core tip:** The role of dietary habits, including consumption of spicy foods, in the development of functional gastrointestinal disorders remains controversial. In this cross-sectional study in a large sample of Iranian adults, we found that women with high consumption of spicy foods had a two-fold increased risk of developing irritable bowel syndrome compared with women who reported not to consume spicy foods. The results underline the need for further studies to characterize potential relationships between diet-related practices and the risk of functional gastrointestinal disorders, in order to design appropriate, and effective, diet-based interventions.

Esmailzadeh A, Keshteli AH, Hajishafiee M, Feizi A, Feinle-Bisset C, Adibi P. Consumption of spicy foods and the prevalence of irritable bowel syndrome. *World J Gastroenterol* 2013; 19(38): 6465-6471 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6465.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6465>

## INTRODUCTION

Functional gastrointestinal disorders (FGIDs) are characterized by chronic and recurrent gastrointestinal (GI) symptoms with ambiguous pathophysiology<sup>[1]</sup>. The most common FGID is irritable bowel syndrome (IBS), which is characterized by abdominal pain and changes in stool consistency and/or frequency<sup>[2-5]</sup>. It has been estimated that 5%-10% of the adult population in Asian countries<sup>[6]</sup> and 10%-20% of the population in developed countries<sup>[7]</sup> are affected by IBS. In Iran, the prevalence of IBS has been reported to be 1.1%-25% based on different studies<sup>[8]</sup>. Since there is no established medical therapy to alter the natural history of IBS in the longer term, the disorder represents a considerable financial burden to the health service, owing to medical consultations and consumption of other valuable resources<sup>[9]</sup>.

Diet appears to play an important role in the etiology of FGIDs<sup>[10-12]</sup>. Dietary intake of carbohydrates and fatty foods along with caffeine, alcohol and spices have been linked to IBS<sup>[13,14]</sup>. Consumption of other foods and nutrients has also been implicated in the induction of symptoms in IBS<sup>[15-20]</sup>. Some studies have suggested that IBS symptoms might result from food sensitivities rather than altered diet composition<sup>[10]</sup>.

The consumption of spicy foods has received attention in relation to FGIDs<sup>[21,22]</sup>. Earlier studies have shown that chili, with its pungent ingredient, capsaicin,

exacerbates abdominal pain and burning in IBS patients. In contrast, chronic consumption of chili has been found to result in an improvement in IBS-related symptoms<sup>[11]</sup>. Six-week administration of four pills per day each containing 150 mg of red pepper powder was reported to be effective in improving the intensity of abdominal pain in IBS patients<sup>[21]</sup>. Other studies have also reported the beneficial effects of spicy foods in the management of FGID symptoms<sup>[22]</sup>. However, most previous studies have focused on chili and its ingredients, and no information is available on other spicy foods. Furthermore, earlier investigations have mostly used high doses of spices as a treatment, and limited data are available examining the habitual consumption of spicy foods and its relationship to the prevalence of IBS.

The traditional Iranian diet contains large amounts of spicy foods, including turmeric, saffron, and ginger, providing an opportunity to assess consumption of spicy foods in relation to health. In addition, few data exist about the association between diet and FGIDs, and available evidence has mostly been reported from small samples, thus, no data are available from large populations. The Study on the Epidemiology of Psychological, Alimentary Health and Nutrition (SEPAHAN), which has been performed in a large group of Iranian adults, provides a unique opportunity to investigate the epidemiological aspects of FGIDs and their relationship with different lifestyles, including nutritional factors<sup>[23]</sup>. Here, we present the sub-study that aimed to explore the association between consumption of spicy foods and the prevalence of IBS among Iranian adults.

## MATERIALS AND METHODS

### Study population

This cross-sectional study was carried out within the framework of the SEPAHAN project. This project was conducted in two main phases in a large sample of Iranian adults working in 50 different healthcare centers across Isfahan province, Iran<sup>[23]</sup>. In the first phase of SEPAHAN, questionnaires on demographic information, medical history, anthropometric measures, lifestyle and nutritional factors were sent to 10087 persons, and 8691 subjects returned the completed questionnaires (response rate: 86.16%). In the second phase, another set of questionnaires was sent out to obtain data on gastrointestinal health of participants. After linking data from both phases and considering missing data, 4763 people who provided complete information on diet and FGIDs were included in the current analysis. The Bioethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran, approved the study.

### Assessment of spicy foods consumption

We used a dietary habits questionnaire that contained detailed questions about meal frequencies, regularity of meals and drinking fluids before and after meals, as well as a question relating to the consumption of spicy foods:

“how often do you use spicy foods (chili pepper, curry, ginger, cinnamon, and turmeric) during a week?” Participants could respond to this question by choosing one of the following choices: never, 1-3 times, 4-6 times, 7-9 times, or more than 10 times per week. Responses to this question were used as the main exposure variable in the current study.

### Assessment of IBS

A modified Persian version of the Rome III questionnaire, as part of the main comprehensive questionnaire, was used for the identification of FGIDs, including IBS<sup>[23]</sup>. During the face validation of the questionnaire, we found that most participants were unable to distinguish between the descriptors used in the original Rome III questionnaire (never, less than one day a month, one day a month, two to three days a month, one day a week, more than one day a week, every day). We, therefore, modified the rating scales to consist of only four descriptors (*i.e.*, never or rarely, sometimes, often, always)<sup>[23]</sup>. Participants were also asked about the presence of each symptom in the previous three months. IBS was defined according to Rome III criteria as recurrent abdominal pain or discomfort at least sometimes in the previous 3 months associated with two or more of the following criteria: (1) improvement with defecation at least sometimes; (2) pain onset associated with a change in stool frequency; and (3) pain onset associated with a change in form (appearance) of stool at least sometimes.

### Assessment of other variables

Standard questionnaires were distributed to collect information on age, gender and educational status. Weight, height and the presence of diabetes mellitus were evaluated by a self-administered questionnaire. Data on smoking were collected through self-reported responses to the questionnaire and participants were categorized as non-smokers, ex-smokers and current smokers. The use of dietary supplements (yes/no) and oral contraceptive pills (OCP) (yes/no) as well as patterns of tea consumption (never or less than 1 cup/mo, 1-3 cups/mo, 1-3 cups/wk, 4-6 cups/wk, 1 cup/d, 2-4 cups/d, 5-7 cups/d, 8-11 cups/d, or at least 12 cups/d) were also assessed by a pre-tested questionnaire. Fluid intake was evaluated through questions on the consumption of water, soft drinks, yogurt drink (“dough”) and other beverages, before, after or during meals, which participants could answer as never, sometimes, often, or always. Regularity of meals was also assessed and quantified as never, sometimes, often, or always having regular meals. Study subjects were also categorized in terms of dental status as fully dentate, lost 1-3 teeth, lost 4-5 teeth, or lost half or more teeth. Quality of chewing was also evaluated (How thoroughly do you chew food?), with responses including: not very well, well, or very well. We also asked participants to describe their feelings/symptoms after milk intake. Lactose intolerance was defined as the existence of abdominal pain, bloating, diarrhea or belching after milk consumption<sup>[24]</sup>.

### Statistical analysis

We categorized participants based on their reported frequency of consumption of spicy foods *i.e.*, never, 1-3 times, 4-6 times, 7-9 times, or 10 times or more during a week. Comparison of continuous variables across different categories of spicy foods consumption was performed using one-way analysis of variance. Distribution of study participants in terms of categorical variables across different categories of intake of spicy foods was compared using  $\chi^2$  test. To assess the relationship between spicy foods consumption and IBS, logistic regression analysis was performed in different models. First, we adjusted for age (continuous) and gender (categorical). We further controlled for smoking (non-smoker, ex-smoker and current smoker), dietary supplement (yes/no) and OCP use (yes/no), self-reported diabetes (yes/no) and body mass index (continuous) in the second model. Additional adjustments were made for meal regularity (never, sometimes, often and always), quality of chewing foods (not very well, well and very well), intra-meal fluid intake (never, sometimes, often and always), dental status (fully dentate, lost 1-3 tooth, lost 4-5 tooth, lost half or more tooth) and pattern of tea consumption (never or < 1 cup/mo, 1-3 cups/mo, 1-3 cups/wk, 4-6 cups/wk, 1 cup/d, 2-4 cups/d, 5-7 cups/d, 8-11 cups/d or at least 12 cups/d). In the final model, a further adjustment was made for lactose intolerance (yes/no). In all analyses, the category of never consuming spicy foods was considered as the reference category. To assess the trend of odds ratios across increasing categories of spicy foods intake, we applied Mantel-Haenszel extension chi-square. A stratified analysis by gender was also performed to examine gender-specific associations. All analysis was performed using SPSS version 16 (SPSS Corp, Chicago, IL, United States). *P* values less than 0.05 were considered statistically significant.

## RESULTS

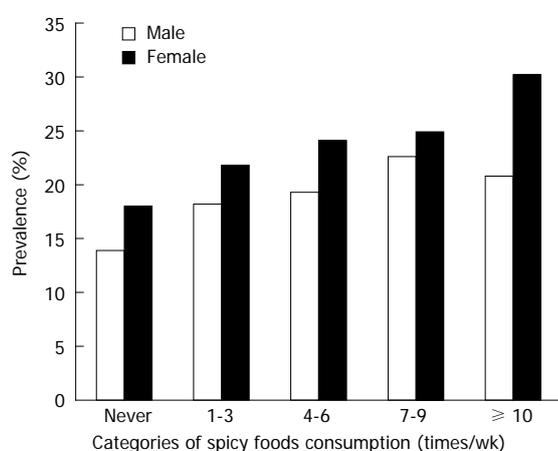
IBS was prevalent among 21.7% (18.6% of men and 24.1% of women) of the study population. General characteristics of study participants across different categories of spicy food consumption are summarized in Table 1. Those consuming spicy foods  $\geq 10$  times/wk were younger, had lower weight and were more likely to be women, married and highly educated compared with those who never consumed spicy foods. High consumption of spicy foods was associated with a lower prevalence of smoking and high prevalence of dietary supplement consumption and OCP use. There was no significant difference in the prevalence of self-reported diabetes among different groups of spicy food intake.

The prevalence of IBS across different categories of spicy foods consumption in the entire study population and each gender is shown in Figure 1. Consumption of spicy foods was associated with an increased prevalence of IBS among women, so that those consuming spicy foods  $\geq 10$  times/wk were more likely to have IBS com-

**Table 1** General characteristics of study participants across different categories of spicy foods consumption

	Consumption of spicy foods (times/wk)					<sup>1</sup> P value
	Never	1-3	4-6	7-9	≥ 10	
Age (yr)	38.0 ± 8.3	37.4 ± 8.4	36.5 ± 7.8	35.1 ± 7.3	33.9 ± 7.6	< 0.001
Weight (kg)	70.3 ± 15.2	70.7 ± 13.4	68.2 ± 12	66.9 ± 14.0	65.4 ± 11.7	< 0.001
BMI (kg/m <sup>2</sup> )	25.1 ± 5.3	25.2 ± 5.0	24.9 ± 4.0	25.0 ± 5.0	24.5 ± 3.8	0.16
Female	38.10%	41.30%	63.50%	73.50%	76.50%	< 0.001
Marriage	78.70%	81.10%	82.70%	79.00%	81.60%	0.03
University degree	44.90%	50.50%	62.00%	65.50%	65.20%	< 0.001
Current smokers	6.30%	4.20%	4.00%	1.40%	1.60%	0.01
Supplement use	4.10%	6.10%	8.30%	9.10%	9.80%	< 0.001
OCP use	1.00%	1.60%	3.00%	4.70%	4.40%	< 0.001
Self-reported diabetes	1.70%	1.80%	1.90%	1.70%	1.40%	0.72

All values are mean ± SD; <sup>1</sup>Obtained from ANOVA or  $\chi^2$  test, as appropriate. BMI: Body mass index; OCP: Oral contraceptive pill.



**Figure 1** Prevalence of irritable bowel syndrome across different categories of spicy food consumption in men and women. Irritable bowel syndrome (IBS) was defined according to the Rome III criteria. Consumption of spicy foods was associated with an increased prevalence of IBS among women ( $P < 0.05$ ). A trend for greater prevalence of IBS with consumption of spicy foods was also seen in men; however, a slight drop in its prevalence was evident in the top category.

pared with those who never consumed spicy foods (30.2% *vs* 18.0%,  $P < 0.05$ ). A trend for greater prevalence of IBS with consumption of spicy foods was also seen in men; however, a slight drop in its prevalence was evident in the top category.

Multivariable adjusted odds ratio (OR) for IBS in the entire study population among different categories of spicy foods intake are illustrated in Table 2. Increased consumption of spicy foods was associated with a greater chance of having IBS in the crude model. After controlling for age and gender, those consuming spicy foods  $\geq 10$  times/wk were two times more likely to have IBS compared with those who never consumed spicy foods. The association remained significant even after taking other cofounders into account (OR = 1.92, 95%CI: 1.23-3.01). Further adjustment for dietary behaviors and lactose intolerance slightly attenuated the association, but it remained significant (OR = 1.85, 95%CI: 1.18-2.90).

Stratified analysis by gender revealed that the association between consumption of spicy foods and IBS

was not significant in men, even after controlling for cofounders (Table 3); however, a significant association was found among women after taking potential cofounders, including meal regularity and lactose intolerance, into account. Those who consumed spicy foods  $\geq 10$  times/wk were twice as likely to have IBS compared with those who never consumed spicy foods (OR = 2.03, 95%CI: 1.09-3.77,  $P_{\text{trend}} = 0.02$ ).

## DISCUSSION

We found that consumption of spicy foods was associated with increased prevalence of IBS among Iranian adults. This association remained significant even after adjustment for potential cofounders, including dietary behaviors. After stratified analysis by sex, the associations remained significant only in women. To the best of our knowledge, our study is among the first population-based studies that assessed habitual intake of spicy foods as a major exposure variable in relation to IBS.

Associations between the consumption of spicy foods and FGIDs, including IBS, have been examined previously<sup>[11,13,14,21,22]</sup>. However, most studies have attempted to use spices to alleviate pain in these patients<sup>[4,25,26]</sup>, and limited data are available on the relationship between habitual consumption of spicy foods and symptoms of IBS<sup>[11]</sup>. Furthermore, prior studies have mostly focused on pepper<sup>[11,13,14,21,22]</sup>, and effects of other spicy foods on IBS remain to be identified. In the current study, we found a significant, and direct, association between consumption of spicy foods and IBS. Our findings are in line with a population-based study in China, which showed a significant association between excessive intake of pepper and prevalence of IBS in adolescents<sup>[14]</sup>. The link between spicy food consumption and IBS symptoms is also supported by an acute, meal-based study<sup>[27]</sup>, in which administration of a standard meal containing 2 g chili, either mixed into the meal or given separately in capsules, caused more abdominal pain and burning in IBS patients than in healthy participants. In contrast, in other studies, ingestion of 4 enteric-coated pills per day (each containing 150 mg of capsaicin-equivalent) for 6 wk, signifi-

**Table 2** Multivariable-adjusted odds ratios and 95%CI for irritable bowel syndrome across different categories of spicy foods consumption<sup>1</sup>

	Consumption of spicy foods (times/wk)					<i>P</i> <sub>trend</sub>
	Never	1-3	4-6	7-9	≥ 10	
Crude	1.00	1.34 (0.95-1.88)	1.57 (1.12-2.21)	1.75 (1.21-2.52)	2.12 (1.45-3.10)	< 0.001
Model I	1.00	1.35 (0.92-1.97)	1.48 (1.00-2.18)	1.58 (1.04-2.39)	1.99 (1.30-3.06)	< 0.001
Model II	1.00	1.28 (0.87-1.88)	1.44 (0.98-2.13)	1.45 (0.95-2.20)	1.94 (1.26-2.98)	< 0.001
Model III	1.00	1.21 (0.81-1.80)	1.35 (0.90-2.01)	1.35 (0.87-2.07)	1.92 (1.23-3.01)	< 0.01
Model IV	1.00	1.20 (0.80-1.78)	1.33 (0.89-1.98)	1.32 (0.86-2.03)	1.85 (1.18-2.90)	< 0.01

<sup>1</sup>Model I : Adjusted for age and gender; Model II : Adjusted for age, gender, body mass index (BMI), smoking, dietary supplements and oral contraceptive pill (OCP) use and self-reported diabetes; Model III: Adjusted for age, gender, BMI, smoking, dietary supplements and OCP use, self-reported diabetes, meal regularity, chewing quality, fluid intakes, pattern of tea consumption and dental status; Model IV: Adjusted for all variables in model III and lactose intolerance.

**Table 3** Multivariable-adjusted odds ratios and 95%CI for irritable bowel syndrome across different categories of spicy foods consumption, stratified by gender

	Consumption of spicy foods (times/wk)					<i>P</i> <sub>trend</sub>
	Never	1-3	4-6	7-9	≥ 10	
<b>Men</b>						
Crude	1.00	1.38 (0.88-2.17)	1.48 (0.92-2.37)	1.81 (1.03-3.16)	1.62 (0.85-3.08)	0.07
Model I	1.00	1.32 (0.79-2.22)	1.34 (0.78-2.31)	1.78 (0.95-3.32)	1.78 (0.87-3.60)	0.10
Model II	1.00	1.27 (0.75-2.14)	1.35 (0.78-2.33)	1.69 (0.89-3.19)	1.8 (0.88-3.67)	0.17
Model III	1.00	1.16 (0.67-2.01)	1.26 (0.71-2.24)	1.55 (0.79-3.04)	1.65 (0.78-3.48)	0.22
Model IV	1.00	1.18 (0.68-2.05)	1.27 (0.71-2.27)	1.55 (0.79-3.04)	1.64 (0.78-3.48)	0.29
<b>Women</b>						
Crude	1.00	1.26 (0.75-2.12)	1.44 (0.87-2.40)	1.5 (0.89-2.55)	1.96 (1.14-3.37)	< 0.01
Model I	1.00	1.38 (0.78-2.43)	1.58 (0.90-2.78)	1.57 (0.87-2.81)	2.14 (1.18-3.88)	< 0.01
Model II	1.00	1.32 (0.75-2.35)	1.53 (0.87-2.68)	1.43 (0.79-2.52)	2.05 (1.12-3.73)	< 0.01
Model III	1.00	1.3 (0.72-2.33)	1.45 (0.81-2.59)	1.38 (0.75-2.52)	2.13 (1.15-3.95)	0.01
Model IV	1.00	1.26 (0.70-2.27)	1.41 (0.79-2.52)	1.34 (0.73-2.44)	2.03 (1.09-3.77)	0.02

Model I : Adjusted for age and gender; Model II : Adjusted for age, gender, body mass index (BMI), smoking, dietary supplements and oral contraceptive pill (OCP) use and self-reported diabetes; Model III: Adjusted for age, gender, BMI, smoking, dietary supplements and OCP use, self-reported diabetes, meal regularity, chewing quality, fluid intakes, pattern of tea consumption and dental status; Model IV: Adjusted for all variables in model III and lactose intolerance.

cantly improved abdominal pain and bloating in IBS patients<sup>[21]</sup>; ginger was found to be the most common type of complementary and alternative medicine used for IBS treatment<sup>[4]</sup>; cinnamon administration has been found to reduce the number of IBS symptoms<sup>[25]</sup>; and beneficial effects of turmeric on abdominal pain and discomfort in IBS patients have also been reported<sup>[26]</sup>. While the causes for the discrepant study outcomes are not clear, there are a number of potential reasons. Different spices may have different modes of action; almost all studies that reported beneficial effects of individual spices used high doses in the form of supplements, and study designs and methodologies varied markedly between studies. It appears that the current study is the first observational study in an adult population, in which habitual consumption of spicy foods has been linked to IBS, although, due to the use of a single question to assess spicy food intake, it is not possible to distinguish between the potential effects of individual spices.

The mechanisms through which consumption of spicy foods might affect IBS are unknown. The effect of red pepper has been related to its pungent ingredient, capsaicin, which can modify gastrointestinal sensation

*via* transient potential vanilloid 1 (TRPV1) receptors<sup>[11,27]</sup>. Increased TRPV1 receptors are associated with visceral hypersensitivity in the proximal gut and colon<sup>[11,27]</sup>. It seems that capsaicin intake in IBS patients can lead to hypersensitivity, which in turn can result in TRPV1 up-regulation<sup>[4,11,28]</sup>. However, few studies have postulated that intermittent and chronic ingestion of capsaicin or capsaicin containing chili can improve FGID symptoms by desensitization of TRPV1 receptors<sup>[11]</sup>. This can be explained by the action of capsaicin, which when administered chronically, depletes nerve terminals of substance P, while acute application leads to maximal release of transmitters, resulting in pain. Further research is required to prove this hypothesis. The mechanisms of other spices remain to be identified.

This study has several strengths. Firstly, it is a large population-based study, which examined habitual intake of spicy foods, rather than the effects of high doses of spices. Earlier studies have mostly been performed in small sample sizes. Secondly, we performed rigorous statistical analyses, including adjustments for several potential contributing factors to IBS. Therefore, the associations we identified are independent of many factors,

including dietary behaviors. Nevertheless, the findings need to be interpreted in the light of some limitations. We used a pre-tested questionnaire for assessing dietary intakes of spicy foods; misclassification is a potential concern in our study as in any epidemiological studies. In addition, high consumption of spicy foods was associated with a complex pattern of lifestyles that may not have been accurately captured and controlled in our analysis, resulting in residual confounding. The significant direct association of spicy foods intake and IBS may be attributed to the other factors (*e.g.*, having irregular meals, not chewing foods very well, *etc.*) associated with higher intake of these foods. That said, the apparently 1 direct effect of spicy food consumption persisted in multivariate models accounting for known potential confounders. Furthermore, some intermediate factors might lead to changes in diet and may, therefore, confound the association between spicy food intake and IBS. In addition, the observed association may not apply to other sections of the Iranian population, including the young, elderly or those from different socio-economic backgrounds. However, participants in the current study were selected from different areas of Isfahan province with diverse socio-economic status and their dietary intakes covered a broad range of dietary habits. Given these characteristics, it is unlikely that this type of bias could explain the observed associations between spicy food intake and IBS.

In conclusion, we found evidence indicating that spicy food consumption was positively associated with IBS, particularly in women. Further studies, in particular of a prospective nature, are required to examine this association in more detail and to potentially develop novel dietary approaches to manage IBS and other FGIDs.

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## COMMENTS

### Background

The relationship between dietary factors and irritable bowel syndrome (IBS) remains yet to be clearly defined. Some research suggests that carbohydrate, protein, fiber, or water all may play a role. However, currently there are many controversial findings regarding the relationship between diet-related practices and different gastrointestinal disorders including IBS.

### Research frontiers

While some studies have indicated that acute chili ingestion can aggravate abdominal pain and burning symptoms in functional gastrointestinal disorders, chronic ingestion of chili was found to improve functional dyspepsia and gastroesophageal reflux disease symptoms in small controlled studies. It is worth noting that most previous studies focused on one type of spice (*e.g.*, chili pepper), and there are few studies that have investigated the relationships between habitual intake of spicy foods and functional gastrointestinal disorders.

### Innovations and breakthroughs

In a large cross-sectional study, information on habitual spicy food intake and symptoms related to IBS were gathered from 4763 adults using standard questionnaires. Individuals with a high intake of spicy foods ( $\geq 10$  times/wk) had an

almost two-fold increased risk of having IBS compared with those who reported a lower intake of spicy foods. After taking into account different variables that might distort the association between spicy food intake and IBS, the relationship was significant only among women.

### Applications

The findings of the current study, if confirmed in well-designed prospective studies, may assist with the design of novel dietary therapies that take into account, and modify, the dietary intake of spicy foods and, thus, may be useful in the management of IBS related symptoms.

### Peer review

In this interesting manuscript, the authors explored the association between consumption of spicy foods and prevalence of IBS among Iranian adults. They performed a cross-sectional study from 4763 Iranian adult participants. Consumption of spicy foods was estimated using a dietary habits questionnaire, and the prevalence of IBS was estimated using a modified Persian version of the Rome III questionnaire. The study has concluded that consumption of spicy foods is directly associated with IBS, particularly in women. This article is interesting and the readers will get some beneficial information from this.

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## Midterm outcome of stapled transanal rectal resection for obstructed defecation syndrome: A single-institution experience in China

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**RESULTS:** The median follow-up was 30 mo (range, 30-46 mo). Late postoperative complications occurred in 11 (14.7%) patients. Three of these patients required procedure-related reintervention (one diverticulectomy and two excision of staple granuloma). Although the recurrence rate was 10.7%, constipation scores (CSS, ODS score and SSS) significantly improved after STARR ( $P < 0.0001$ ). Significant reduction in ODS symptoms was matched by an improvement in the PAC-QOL and VAS ( $P < 0.0001$ ), and the satisfaction index was excellent in 25 (33.3%) patients, good in 23 (30.7%), fairly good in 14 (18.7%), and poor in 13 (17.3%). Nevertheless, the WS increased after STARR ( $P = 0.0169$ ). Incontinence was present or deteriorated in 8 (10.7%) patients; 6 (8%) of whom were new onsets. Univariate analysis revealed that the occurrence of fecal incontinence (preoperative, postoperative or new-onset incontinence;  $P = 0.028, 0.000, \text{ and } 0.007$ , respectively) was associated with the success of the operation.

**CONCLUSION:** STARR is an acceptable procedure for the surgical correction of ODS. However, its impact on symptomatic recurrence and postoperative incontinence may be problematic.

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**Key words:** Colorectal surgery; Constipation; Stapled transanal rectal resection

### Abstract

**AIM:** To assess midterm results of stapled transanal rectal resection (STARR) for obstructed defecation syndrome (ODS) and predictive factors for outcome.

**METHODS:** From May 2007 to May 2009, 75 female patients underwent STARR and were included in the present study. Preoperative and postoperative workup consisted of standardized interview and physical examination including proctoscopy, colonoscopy, anorectal manometry, and defecography. Clinical and functional results were assessed by standardized questionnaires for the assessment of constipation constipation scoring system (CSS), Longo's ODS score, and symptom severity score (SSS), incontinence Wexner incontinence score (WS), quality of life Patient Assessment of Constipation-Quality of Life Questionnaire (PAC-QOL), and patient satisfaction visual analog scale (VAS). Data were collected prospectively at baseline, 12 and 30 mo.

**Core tip:** As a less-invasive surgical procedure, stapled transanal rectal resection (STARR) is becoming an important option in the treatment of obstructive defecation syndrome. However, its clinical and functional outcomes are still conflicting and controversial. The present study assessed the midterm results after STARR performed by the same team in our department to identify factors for predicting outcome. Our data provide evidence to attest the clinic benefits of this pro-

cedure, but its impact on symptomatic recurrence and postoperative incontinence may be problematic.

Zhang B, Ding JH, Zhao YJ, Zhang M, Yin SH, Feng YY, Zhao K. Midterm outcome of stapled transanal rectal resection for obstructed defecation syndrome: A single-institution experience in China. *World J Gastroenterol* 2013; 19(38): 6472-6478 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6472.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6472>

## INTRODUCTION

Obstructed defecation syndrome (ODS) is defined as the normal desire to defecate but with an impaired ability to evacuate the rectum satisfactorily<sup>[1]</sup>. The anatomical and physiological disturbances underlying ODS are complex and only partly understood, but rectocele and intussusception have been identified as the two most important organic causes of ODS<sup>[2]</sup>.

Although a variety of surgical approaches has been described in the literature for correction of ODS, most of these have high recurrence and complication rates. Stapled transanal rectal resection (STARR) was introduced in 2003 by Longo<sup>[3]</sup> as a minimally invasive transanal operation for ODS associated with rectocele and intussusception. The novel procedure is carried out using double circular stapler devices to resect a full-thickness segment of rectal wall and subsequently to restore normal rectal anatomy. In contrast to traditional techniques, STARR addresses correction of both rectocele and intussusception.

Several multicenter trials have demonstrated that STARR significantly improves constipation with low morbidity and high comfort for patients<sup>[4-8]</sup>. In addition, the procedure could even offer long-term clinical benefits<sup>[9-11]</sup>. Nevertheless, worrisome complications and unsatisfactory functional results have been described<sup>[12,13]</sup>. There are also reports of high rates of reintervention for both symptomatic recurrence and procedure-related complications after this surgery<sup>[14,15]</sup>. As a consequence, although STARR is increasingly being accepted as an important option for surgical treatment of ODS, its clinical and functional outcomes are still conflicting and controversial.

We have shown previously that STARR can be performed safely and is effective for eligible patients with ODS secondary to rectocele and intussusceptions<sup>[16,17]</sup>. The objective of this study was to assess midterm clinical and functional results and to identify factors for predicting outcome after STARR.

## MATERIALS AND METHODS

### Patients

From May 2007 to May 2009, a consecutive series of 86 female patients was treated with STARR for ODS in our Department of Colorectal Surgery at the Second Artillery General Hospital, Beijing, China. A total of 75 (87.2%)

patients completed the scheduled follow-up and formed the study population. All patients were prospectively included in a database. Study protocol was approved by the institutional ethics committee of our hospital. Written informed consent was obtained from all patients enrolled in the study. Preoperative assessment included symptom evaluation, clinical and gynecological examinations, and investigations with proctoscopy, colonoscopy, colonic transit time study, anorectal manometry, and defecography. Anorectal manometry was performed as previously described<sup>[17]</sup>. Patients were carefully selected according to the inclusion and exclusion criteria for STARR proposed by the consensus recommendations<sup>[18]</sup> and the decision-making algorithm<sup>[2]</sup>.

### Surgical procedures

Polyethylene glycol electrolyte solutions were preoperatively prescribed for bowel preparation. Patients received routine broad-spectrum antibiotics immediately after anesthesia induction. Under spinal anesthesia, patients were placed in the lithotomy position with a catheter in the bladder. The STARR procedure was performed using the circular stapler (PPH-01; Ethicon Endo-Surgery, Inc., New Brunswick, NJ, United States) as described previously<sup>[4]</sup>. Subsequent bleeding from the staple line was controlled with full-thickness 2-0 Vicryl stitches (Ethicon Endo-Surgery). All STARR procedures were conducted by the same surgical team.

### Outcome measures

The severity of ODS was quantified by the validated constipation scoring system (CSS; range: 0-30 at increments of 1; no symptoms = 0)<sup>[19]</sup>; Longo's ODS score (range: 0-40 at increments of 1; no symptoms = 0)<sup>[16]</sup>; and symptom severity score (SSS; range: 0-36 at increments of 1; no symptoms = 0)<sup>[7]</sup>. Patient's fecal incontinence was assessed by the Wexner incontinence score (WS; range: 0-36 at increments of 1; perfect continence = 0)<sup>[20]</sup>. The validated Patient Assessment of Constipation-Quality of Life Questionnaire (PAC-QOL) was used to measure the quality of life in patients with ODS<sup>[21]</sup>. The first three subscales of the self-reported questionnaire were used to assess the patient dissatisfaction index, with an overall score ranging from 0 to 96 (lower scores corresponding to better quality of life). The satisfaction subscale included four items with a global score ranging from 0 to 16 (high scores corresponding to better quality of life)<sup>[22]</sup>. Moreover, the index of patient satisfaction was also measured by the visual analog scale (VAS) with scores from 0 to 10, and a higher score suggested an improvement in patient satisfaction with surgery.

### Postoperative follow-up

The patients were followed up in our clinic at 3, 6, 12 and 30 mo postoperatively. At each visit, digital rectal examination was used to assess the anal sphincter, and proctoscopy or colonoscopy to evaluate the anastomosis and the presence or absence of local complications (stenosis,

**Table 1 Univariable analysis of predictive factors correlated with therapy success after stapled transanal rectal resection**

Factors	Total (n = 75)	Successful (n = 62)	Unsuccessful (n = 13)	P value
Mean age (yr) <sup>1</sup>	54.30	53.80	56.50	0.287
Multiparous/non-multiparous <sup>2</sup>	31/44	24/38	7/6	0.314
Hysterectomy/no hysterectomy <sup>3</sup>	10/65	7/55	3/10	0.364
Anorectal operation before STARR/no operation <sup>2</sup>	36/39	29/33	7/6	0.643
Constipation scores <sup>1</sup>				
CSS score	15.57	15.60	15.46	0.569
ODS score	18.39	18.03	20.08	0.994
SSS score	13.69	13.55	14.38	0.537
Manometric parameters <sup>1</sup>				
Resting pressure (mmHg)	54.13	54.27	53.46	0.497
Squeeze pressure (mmHg)	109.0	109.5	106.7	0.726
First initial sensation (mL)	87.05	86.53	89.54	0.649
Maximum tolerable rectal volume (mL)	238.2	238.2	238.0	0.248
Defecographic parameters				
Rectocele (mm) <sup>1</sup>	35.12	34.62	37.46	0.220
Intussusception/no intussusception <sup>3</sup>	65/10	56/6	9/4	0.064
Increased perineal descent/no perineal descent <sup>3</sup>	21/54	15/47	6/7	0.171
Sigmoidocele/no sigmoidocele <sup>3</sup>	9/66	7/55	2/11	0.650
Fecal incontinence <sup>3</sup>				
Preoperative incontinence/no incontinence	2/73	0/62	2/11	0.028
Postoperative incontinence/no incontinence	8/67	2/60	6/7	0.000
New-onset incontinence/no incontinence	6/69	2/60	4/9	0.007

<sup>1</sup>Unpaired *t* test; <sup>2</sup>Pearson's  $\chi^2$  test; <sup>3</sup>Fisher's exact test. STARR: Stapled transanal rectal resection; CSS: Constipation scoring system; SSS: Symptom severity score; ODS: Obstructed defecation syndrome.

granulomas or mucosal prolapse). We also recorded the occurrence of postoperative complications, which were considered to be early if they occurred within 1 mo after surgery and late if they occurred after this period. A complete clinical reassessment including anorectal manometry and defecography was performed at 12 mo after surgery. Functional results were further updated at 30 mo of follow-up using the same standardized questionnaires (CSS, ODS score, SSS, WS, PAC-QOL and VAS). The STARR procedure was considered successful at 30 mo when PAC-QOL (satisfaction index) scores were classified as excellent, good, or fairly good, defined as follows: 13-16 classified as excellent, 9-12 as good, 5-8 as fairly good, and 0-4 as poor.

### Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows XP (SPSS Inc. Chicago, IL, United States). The variation of total scores of the CSS, ODS, SSS, WS, PAC-QoL and VAS were expressed as mean values with 95%CI. Data were compared between groups using the two-sample *t* test, paired *t* test, Pearson's  $\chi^2$  test, Fisher's exact test, and Wilcoxon signed-rank test, as indicated. *P* < 0.05 was considered statistically significant.

## RESULTS

### Preoperative data

Of the 75 female patients (mean age, 54.3 years; range, 29-75 years) included in this study, 60 (80%) had experienced vaginal delivery and 31 (41.3%) were multiparous. Sixty-four (61.3%) patients underwent previous anorec-

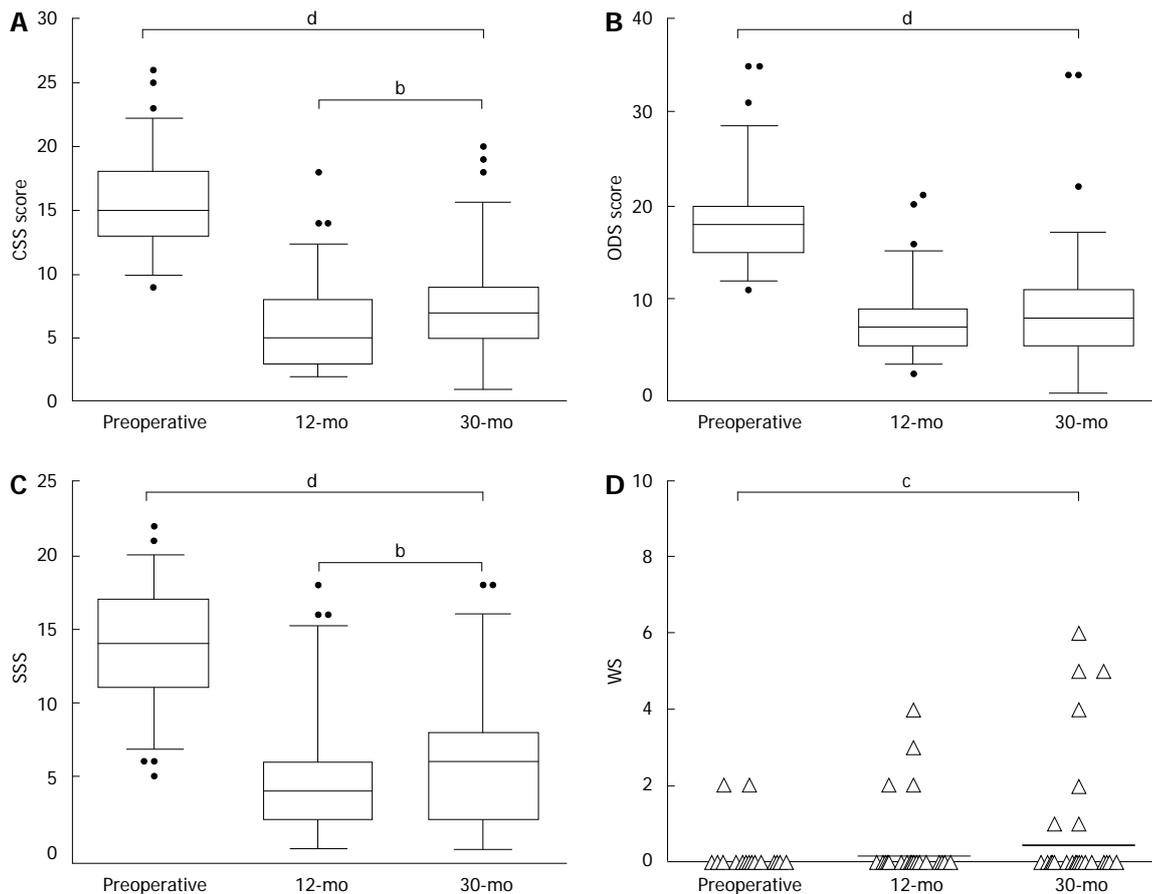
tal/gynecological surgery, including episiotomy (18 patients), hemorrhoidectomy (14 patients), fistulectomy (3 patients), sphincterotomy (1 patient), and hysterectomy (10 patients). Defecographic and manometric findings are detailed in Table 1.

### Perioperative data

A staple-line dehiscence necessitating handsewn suturing was the only intraoperative complication that we observed. There were no major complications, rectovaginal fistula, pelvic sepsis, or deaths. The operative data, early postoperative complications, and short-term results were described in our previous studies<sup>16,17</sup>.

### Late postoperative complications

A total of 12 late complications occurred in 11 patients, giving an overall morbidity rate of 14.7%. The most frequently reported complication was postoperative incontinence, which was present or deteriorated in eight (10.7%) patients. Although defecatory urgency vanished spontaneously in most patients within the first 3 mo postoperatively, one (1.3%) patient reported this complaint at the time of the latest interview. Two (2.7%) patients suffered from inflammatory granulomas on the staple line, which had to be removed because of chronic pain or bleeding. Additionally, there was one (1.3%) case of iatrogenic rectal diverticulum with impacted fecalith confirmed 34 mo after surgery. It presented as severe recurrence of obstructed defecation and was treated by transanal diverticulectomy<sup>123</sup>. Thus, 3 (4%) patients required transanal reintervention for procedure-related complications after STARR.



**Figure 1** Preoperative and postoperative assessment in 75 patients with obstructed defecation syndrome undergoing stapled transanal rectal resection. A: CSS scores (paired *t* test, <sup>b</sup>*P* < 0.01, <sup>d</sup>*P* < 0.01); B: Longo's ODS scores (paired *t* test, <sup>d</sup>*P* < 0.01); C: SSS (paired *t* test, <sup>b</sup>*P* < 0.01, <sup>d</sup>*P* < 0.01); D: WS (paired *t* test, <sup>c</sup>*P* < 0.05). CSS: Constipation scoring system; ODS: Obstructed defecation syndrome; SSS: Symptom severity score; WS: Wexner incontinence scores.

### Follow-up data

Changes in the constipation scores (CSS, ODS score and SSS) and the incontinence scores (WS) are presented in Figure 1. Globally, a significant reduction in the CSS, ODS score and SSS was observed at 12 mo as compared with baseline, and this reduction was maintained at 30 mo [CSS at baseline *vs* 30 mo: 15.57 (95%CI: 14.78-16.36) *vs* 7.07 (95%CI: 6.16-7.98); ODS score: 18.39 (95%CI: 17.27-19.51) *vs* 8.55 (95%CI: 7.12-9.97); SSS: 13.69 (95%CI: 12.74-14.64) *vs* 6.16 (95%CI: 5.12-7.20); *P* < 0.0001 in each group]. However, these scores started to increase slightly after 12 mo [CSS at 12 mo *vs* 30 mo: 5.99 (95%CI: 5.28-6.70) *vs* 7.07 (95%CI: 6.16-7.98); SSS: 4.59 (95%CI: 3.73-5.45) *vs* 6.16 (95%CI: 5.12-7.20), *P* < 0.01; ODS score: 7.49 (95%CI: 6.65-8.34) *vs* 8.55 (95%CI: 7.12-9.97), *P* = 0.07]. Overall, the symptoms of ODS had persisted or recurred in 8 (10.7%) patients with adequate follow-up. Two patients who had initial improvement presented with persistence of ODS symptoms 3 mo after surgery, and another 6 patients developed symptomatic recurrence after 12 mo.

Although the WS rose slightly after STARR, two cases of new-onset fecal incontinence and two of worsened incontinence were observed during 12 mo follow-up, there was no significant difference before and after surgery [WS

at baseline *vs* at 12 mo: 0.05 (95%CI: -0.02-0.13) *vs* 0.15 (95%CI: -0.003-0.30), *P* = 0.052]. However, another four patients had new-onset incontinence after 12 mo and the WS increased significantly at 30 mo follow-up [WS at baseline *vs* at 30 mo: 0.05 (95%CI: -0.02-0.13) *vs* 0.43 (95%CI: 0.09-0.76), *P* = 0.017]. On the whole, incontinence was present or deteriorated in 8 (10.7%) patients, 6 (8%) of whom had new onset.

As shown in Table 2, improvement in the constipation scores was matched by an overall improvement in quality of life as assessed by the PAC-QOL and VAS scores at both 12 and 30 mo follow-up [PAC-QOL (dissatisfaction index) at baseline *vs* 30 mo: 44.45 (95%CI: 41.15-47.76) *vs* 13.21 (95%CI: 10.36-16.07); PAC-QOL (satisfaction index): 0 *vs* 10.12 (95%CI: 9.21-11.03); VAS: 3.83 (95%CI: 3.54-4.11) *vs* 7.07 (95%CI: 6.69-7.46); *P* < 0.0001]. At the end of follow-up, the self-reported definitive outcome was reported as excellent by 25 (33.3%) patients, good by 23 (30.7%), fairly good by 14 (18.7%), and poor by 13 (17.3%). Symptomatic recurrence and postoperative incontinence were the main reasons for a poorer outcome.

### Predictive factors for outcome

In accordance with the patient's assessment of the clini-

**Table 2** Preoperative and postoperative scores of quality-of-life questionnaires and visual analog scale in 75 patients undergoing stapled transanal rectal resection

Items	Median		
	Preoperative	12 mo	30 mo
PAC-QoL (dissatisfaction index)	44	7 <sup>b</sup>	9 <sup>b</sup>
PAC-QoL (satisfaction index)	0	12 <sup>b</sup>	10 <sup>b</sup>
VAS satisfaction index	4	8 <sup>b</sup>	7 <sup>b</sup>

The Wilcoxon signed-rank test was used; All the comparisons *vs* the preoperative data were statistically significant; <sup>b</sup>*P* < 0.01. PAC-QoL: Postoperative scores of quality-of-life questionnaires; VAS: Visual analog scales.

cal outcome at 30 mo follow-up, 17 patient- and disease-related factors were used to compare 65 patients who acquired any improvement after STARR with 13 patients who considered an absence of success for further statistical analyses (Table 1). The result of the univariate analysis revealed that lack of improvement was more likely in patients with fecal incontinence (preoperative, postoperative or new-onset incontinence; *P* = 0.028, 0.000, and 0.007, respectively). However, multiparous, hysterectomy, previous anorectal operation, CSS, ODS score, SSS, and defecographic or manometric findings were not correlated with the functional success of the operation.

## DISCUSSION

Controversy exists in the literature regarding the results after STARR, therefore, this study aimed to evaluate the midterm results and predictive factors for outcome. We assessed a series of 75 patients before and 30 mo after STARR, in which late postoperative complications were seen in 14.7% and reintervention was required in 4%. Despite the recurrence rate of 10.7%, clinical and functional outcome scores (CSS, ODS, SSS, PAC-QOL, and VAS) significantly improved after surgery. Nevertheless, the significant reduction in ODS symptoms was not matched by impairment of the WS. The success of the STARR procedure was associated with the occurrence of fecal incontinence, which was present or deteriorated in 10.7% of patients after surgery.

Several studies have indicated the midterm efficacy of STARR in relieving ODS symptoms with high patient satisfaction rates<sup>[4,5,24-27]</sup>. Similar clinic benefits were obtained in the present study; we were able to demonstrate that defecation difficulties were significantly improved after STARR. Improvement remained stable at 30 mo follow-up as compared with baseline, albeit the constipation scores started increase 12 mo after surgery. Meanwhile, the satisfaction index was reported as excellent in 25 (33.3%), good in 23 (30.7%), fairly good in 14 (18.7%), and poor in 13 (17.3%). Hence, our midterm follow-up suggests that early postoperative benefits were maintained. Other reports, however, showed that ODS symptoms may not improve or even deteriorate after STARR<sup>[13,14]</sup>. The main reason for these conflicting observations may be the patient selection criteria. Inadequate

indications for this operation will necessarily result in poor outcome. The outcomes of an Italian multicenter study were worse in none-selected patients and improvement after STARR was noted in only 65% of the patients<sup>[14]</sup>. In our series, all patients were carefully selected on the basis of the consensus recommendations and the decision-making algorithm<sup>[2,18]</sup>, but further observations should evaluate whether the midterm efficacy deteriorates with time.

Although STARR produced good midterm results, eight (10.7%) patients in our study presented with persistent or recurrent symptoms of ODS. In the literature, the incidence of midterm recurrences is between 4.3% and 17.1%<sup>[5,8,14,28]</sup>. More recently, however, it has been shown that none of the patients who underwent STARR by the curved Contour Transtar stapler had recurrence of ODS symptoms during a 36-mo follow-up<sup>[29]</sup>. This discrepancy may be attributed to the limited capacity of PPH-01 casing with risk of leaving residual disease, especially in patients with large rectocele and intussusception. It should also be stressed that rectocele and intussusception are only the emerging tip of the ODS iceberg syndrome; pelvic floor pathology caused by the “underwater rocks” or occult lesions are likely to persist and contribute to persistent or recurrent symptoms after surgery<sup>[30]</sup>.

Some series therefore have been designed to define predictive factors for outcomes after STARR. Gagliardi *et al*<sup>[14]</sup> have suggested that the results were worse in patients with preoperative digitation, puborectalis dyssynergia, enterocele, larger rectocele, lower bowel frequency, and sense of incomplete evacuation. Contrary to this observation, a subsequent study showed that the number of pelvic floor changes was associated with the success of the operation<sup>[11]</sup>. Another study demonstrated that factors for an unfavorable outcome after STARR included small rectal diameter, low sphincter pressure, and increased pelvic floor descent<sup>[8]</sup>. In the present study, we only indicated that the occurrence of fecal incontinence, including preoperative, postoperative or new-onset incontinence, was associated with poorer midterm outcome. In addition, postoperative incontinence was one of the main reasons for patient dissatisfaction. No doubt more evidence is needed to clarify this issue.

Fecal incontinence after STARR is one of the main concerns of surgeons. Postoperative incontinence and urgency have been reported as being transient and disappeared within 6 mo<sup>[4]</sup>, but were still present after 30 mo in some of our patients. Incontinence may be caused by reduced rectal volume or by muscle stretching and transient sphincter dysfunction secondary to the 36-mm dilator<sup>[4,31]</sup>. We did not systematically evaluate the anal sphincter using ultrasound, but there was no evidence of sphincter dysfunction according to our manometry results. Intriguingly, 6 (8%) patients in our study had new-onset incontinence after the STARR procedure. A possible explanation is that intussusception in the anal canal may function as a barrier with a subsequently beneficial effect on fecal continence. After its removal, fecal incontinence becomes

uncovered<sup>[31]</sup>. Consequently, a careful patient selection with the awareness of occult incontinence is crucial. It is noteworthy that incontinence improves in some patients, which is attributed to improved internal sphincter function after STARR<sup>[6,7,25,28]</sup>. Few patients with preoperative incontinence were enrolled, thus, it could not be assessed in our study.

In the current study, STARR was confirmed as a safe procedure for the treatment of ODS. Nevertheless, an unexpected major complication was observed in one patient who developed an iatrogenic rectal diverticulum after STARR. Concordant with previous findings<sup>[12,32]</sup>, the diverticulum was located along the lateral wall of the rectum, an area of weakness, where anterior and posterior suture lines cross over one another. Iatrogenic diverticulum may also occur as a consequence of technical failure in that the lateral part of the rectal wall remained outside the staple casing during the second resection, or an incomplete section of the mucosal band was retained after STARR<sup>[32]</sup>. To the best of our knowledge, no patient has developed rectal diverticulum after Transtar for the surgical correction of ODS; therefore, this major complication may be the inherent drawbacks of the PPH-01 stapler that could be avoided by using the new device.

We conclude that STARR may be an acceptable procedure for the treatment of patients with ODS caused by rectocele and intussusception, but its impact on symptomatic recurrence and postoperative incontinence may be problematic. In this study, patients were strictly selected and systematically assessed prospectively. However, there were still some limitations such as the lack of a control group. Moreover, postoperative defecography or magnetic resonance imaging with longer follow-up is also crucial for providing more details on pelvic floor anatomy as well as physiology. Finally, this was a midterm follow-up study. Further studies are needed to assess long-term results and to optimize patient selection, which is required to enhance and maintain patient satisfaction after surgery.

## COMMENTS

### Background

Obstructed defecation syndrome (ODS) is a frequent but multifactorial disease that usually afflicts middle-aged women. Although a variety of surgical procedures has been proposed to correct ODS, no one has found unanimous consensus. Stapled transanal rectal resection (STARR) was recently introduced as a minimally invasive transanal procedure, the advantage of which is the simultaneous treatment of rectocele and rectal intussusception, both representing the main anatomical cause of ODS.

### Research frontiers

In recent years, STARR is increasingly being accepted as an important option for surgical treatment of ODS. However, the clinical and functional outcomes after STARR are still conflicting and controversial. In the area of treatment of ODS by the STARR procedure, the research hotspots are how to optimize patient selection and to predict the functional outcome after surgery.

### Innovations and breakthroughs

The authors assessed midterm results and predictive factors for outcome after STARR. Even though the recurrence rate was 10.7%, the clinical and functional outcome scores significantly improved after surgery. In addition, symptomatic recurrence and postoperative incontinence were the main reasons for a poorer

outcome.

### Applications

The study results suggest that STARR may be an acceptable procedure for the treatment of ODS, but its impact on symptomatic recurrence and postoperative incontinence may be problematic.

### Peer review

This study assessed the midterm outcome of STARR for ODS. This topic has been previously studied, and the results of several studies have been discordant. Nevertheless, the topic is interesting for the readers of the journal and suitable to be published.

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## A new endoscopic ultrasonography image processing method to evaluate the prognosis for pancreatic cancer treated with interstitial brachytherapy

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### Abstract

**AIM:** To develop a fuzzy classification method to score the texture features of pancreatic cancer in endoscopic ultrasonography (EUS) images and evaluate its utility in making prognosis judgments for patients with unresectable pancreatic cancer treated by EUS-guided interstitial brachytherapy.

**METHODS:** EUS images from our retrospective database were analyzed. The regions of interest were drawn, and texture features were extracted, selected, and scored with a fuzzy classification method using a C++ program. Then, patients with unresectable pancreatic cancer were enrolled to receive EUS-guided iodine 125 radioactive seed implantation. Their fuzzy

classification scores, tumor volumes, and carbohydrate antigen 199 (CA199) levels before and after the brachytherapy were recorded. The association between the changes in these parameters and overall survival was analyzed statistically.

**RESULTS:** EUS images of 153 patients with pancreatic cancer and 63 non-cancer patients were analyzed. A total of 25 consecutive patients were enrolled, and they tolerated the brachytherapy well without any complications. There was a correlation between the change in the fuzzy classification score and overall survival (Spearman test,  $r = 0.616$ ,  $P = 0.001$ ), whereas no correlation was found to be significant between the change in tumor volume ( $P = 0.663$ ), CA199 level ( $P = 0.659$ ), and overall survival. There were 15 patients with a decrease in their fuzzy classification score after brachytherapy, whereas the fuzzy classification score increased in another 10 patients. There was a significant difference in overall survival between the two groups (67 d vs 151 d,  $P = 0.001$ ), but not in the change of tumor volume and CA199 level.

**CONCLUSION:** Using the fuzzy classification method to analyze EUS images of pancreatic cancer is feasible, and the method can be used to make prognosis judgments for patients with unresectable pancreatic cancer treated by interstitial brachytherapy.

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**Key words:** Digital image processing; Fuzzy classification; Endoscopic ultrasonography; Pancreatic cancer; Interstitial brachytherapy; Prognosis

**Core tip:** Digital image processing (DIP) of endoscopic ultrasonography (EUS) images has been proven to be useful in diagnosis of malignant tumor. Currently commonly used method of DIP is only to concludes the dif-

ferential diagnosis of solid tumors (“yes” or “no”), can not provide the numerical data describing the texture parameters in the EUS image. EUS-guided brachytherapy has been applied preliminarily in the study of advanced pancreatic cancer. However, prognosis judgment of these patients was still difficult. So we develop a fuzzy classification method to score texture features of pancreatic cancer in EUS images to supply more information and validated its utility in prognosis judgment of patients with unresectable pancreatic cancer treated by EUS-guided interstitial brachytherapy.

Xu W, Liu Y, Lu Z, Jin ZD, Hu YH, Yu JG, Li ZS. A new endoscopic ultrasonography image processing method to evaluate the prognosis for pancreatic cancer treated with interstitial brachytherapy. *World J Gastroenterol* 2013; 19(38): 6479-6484 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6479.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6479>

## INTRODUCTION

The application of digital image processing (DIP) in endoscopic ultrasonography (EUS) images and other imaging scenarios has been proven to be a useful adjunct to endoscopic diagnoses and often comparable with specialists’ interpretation in different pathologic settings<sup>[1-3]</sup>. The texture parameters of EUS images are extracted and classified from the returned echoes to identify the tissue type present in the images. One effective approach is to use DIP based on a support vector machine (SVM), which is a computer algorithm that learns by example to assign labels to objects<sup>[4,5]</sup>. The SVM technique, as a subfield of digital signal processing, has been applied to a series of pathologically proven diseases<sup>[6-12]</sup>.

The typical method of SVM, which is only able to provide a differential diagnosis for solid tumors (“yes” or “no”), cannot provide numerical data describing the texture parameters in the EUS image. In this study, a new DIP method based on fuzzy classification is applied to obtain the feature value of texture parameters in EUS images of pancreatic cancer and observe the change of texture parameters to evaluate its utility in making prognosis judgments for patients with unresectable pancreatic cancer after EUS-guided interstitial brachytherapy.

## MATERIALS AND METHODS

The whole study protocol was approved by the Institutional Review Board and Ethics Committees of the Second Military Medical University. All patients had provided their written informed consent before the study. DIP of EUS images using the fuzzy classification method was retrospective, whereas its application in the prognosis evaluation was prospective.

### Principle of fuzzy classification

Given that the unidentified object  $u$  had  $p$  classes, which

meant there were  $p$  cases that such an object could be classified to, a number of features were extracted from the object  $u$ , and the sum of these features had a membership degree  $\mathcal{A}$  to every class. Therefore, the membership degree of the unidentified object  $u$  to each class was  $\mathcal{A}_1(u), \mathcal{A}_2(u), \dots, \mathcal{A}_p(u)$ . It is generally assumed that the larger the membership degree’s value of a certain class is, the greater the feature value of the objects belonging to this class will be.

Given that the  $j$ th feature extracted from the unidentified object  $u$  was  $u_j$ , its membership degree to the  $j$ th feature of class  $i$  was:

$$\mathcal{A}_{ij}(u) = (1 + (u_j - a_{ij})^2 / \sigma_{ij}^2)^{-1} \quad (1)$$

where  $a_{ij}$  was the  $j$ th feature’s mean value for the training data belonging to class  $i$ , and  $\sigma_{ij}$  was the variance.

Thus, every feature of the unidentified object  $u$  could obtain a membership degree to class  $i$ . In addition, a corresponding weight  $\alpha_j$  was also assigned to it. Therefore, the membership degree of belonging to class  $i$  should be:

$$\mathcal{A}_i(u) = \left| \sum_{j=1}^m (\alpha_j \times \mathcal{A}_{ij}(u)) \right| \quad (2)$$

The weights could be optimized by taking advantage of the training data.

In terms of the application object in this study, there were two classes: pancreatic cancer and non-pancreatic cancer. For an unidentified case, its membership degree to the two categories  $\mathcal{A}_1$  and  $\mathcal{A}_2$  was computed, and then the feature value was obtained according to the following normalized evaluation function:  $Eval = [\mathcal{A}_1 / (\mathcal{A}_1 + \mathcal{A}_2)] \times 100\%$ . The object is more likely to be a cancer as the feature value gets closer to 100%, and vice versa. Thus, the fuzzy classification of pancreatic cancer was achieved.

### Processing of the fuzzy classification method

The analysis database of EUS images was compiled from data collected between March 2005 and December 2007, which was described in a previous study of our group<sup>[14]</sup>. All EUS procedures were performed with an Olympus GF-UM2000 at 7.5 MHz. Regions of interest (ROIs) of all EUS images were manually outlined by endoscopic specialists who were blind to the final diagnosis. Texture features were extracted from every ROI and analyzed using a C++ program. Texture features generally referred to the spatial arrangement and interconnection of the basic elements of images<sup>[15]</sup>. The sequential forward search algorithm was applied to select the features after extracting the feature. Then, a few optimum feature combinations were obtained<sup>[16,17]</sup>. Finally, real time was taken into account, and 22 features falling into three categories were selected. First, the mean feature value of the image was extracted. It was a first-order statistical feature. Second, the gray level co-occurrence matrix (GLCM) features were selected, which were based on the second-order joint feature distribution matrix of the images proposed by Haralick *et al*<sup>[18]</sup>. GLCMs for four directions (0°, 45°, 90°, and 135°) were constructed. For each matrix, five features were extracted, which were energy, entropy, moment of inertia, correlation, and local stationary. Finally,

the fractal feature was obtained. Recently, the fractal dimension feature has been widely used in pattern recognition and texture analysis. In this study, the second-order multi-fractal dimension feature was used, and the differential box-counting approach was applied to calculate the fractal dimension<sup>[19,20]</sup>. The previously described fuzzy classification method assessed all the features contained in the ROI of the EUS image and estimated a score between 0 and 100. Given that two states existed - cancer and a normal pancreas - 0 represented all the features of a “normal pancreas” that were contained in the ROI with no “cancer” features, whereas 100 represented all the features of “cancer” with no “normal pancreas” features.

### Application of the fuzzy classification method

Written informed consent for EUS-guided interstitial brachytherapy (EUS-guided iodine 125 radioactive seed implantation) was required from all included patients. Patient eligibility criteria included histologically confirmed unresectable pancreatic adenocarcinoma. To be included in the study, patients had to have a Karnofsky performance status score  $\geq 60$  and be expected to survive for more than 2 mo after diagnosis; in addition, they had to exhibit adequate bone-marrow function (absolute neutrophil count  $\geq 1.5 \times 10^9$  cells/L, platelet count  $\geq 100 \times 10^9$ /L, and hemoglobin  $\geq 100$  g/L), kidney function (serum creatinine  $\leq 132.6$   $\mu$ mol/L), and a prothrombin time within 3 s of the control. Exclusion criteria included the inability to give informed consent. Abdominal pain and other accompanying diseases had to be controlled in all patients before inclusion in the study. While receiving implantation treatment, the patients received other necessary treatments such as chemotherapy or biological therapy. The procedure used for radioactive seed implantation was the same one detailed in our previous description<sup>[21]</sup>.

All patients received repeated EUS before and after brachytherapy. All images were reviewed by endoscopists who were blinded to the prognosis. A total of 10 EUS images, 5 images each before and after brachytherapy, were chosen for each patient. The boundary of the ROI was manually delineated, and all the feature values within the ROIs were averaged together. By setting the appropriate range for the estimated scores, the influences of necrotic tissue and radioactive seeds on the calculation results were avoided. The fuzzy classification method calculated two scores for every patient.

All patients were evaluated by weekly physical examinations, complete blood counts, and chemistry profiles. The serum level of carbohydrate antigen 199 (CA199) was measured every 3 wk after the therapy. Standard WHO response criteria were used to define the best anti-tumor effects, toxicities, complications, and adverse events<sup>[22]</sup>. Tumor assessment by the same endoscopic expert with an EUS scan was required every 3 mo. The largest and smallest diameters were recommended to be measured by EUS, and the tumor volume was estimated according the following formula:  $V = 1/2 ab^2$ , where  $a$  and  $b$  are the largest and smallest tumor diameters, re-

spectively, and  $V$  is the tumor volume. Overall survival (OS) was calculated from the day of treatment until the date of death.

### Statistical analysis

The descriptive results of continuous variables were presented as the median (interquartile range, IQR). The relationships between the change of fuzzy classification score, tumor volume, CA199 level, and overall survival were assessed using the Spearman rank correlation test. The patients were divided into two groups according to an increase or decrease in the fuzzy classification score. The overall survival rates of the two groups were compared using the log-rank test. The inter-group comparison of the change of tumor volume and CA199 level was conducted by a Mann-Whitney  $U$  test. The results were considered statistically significant at  $P < 0.05$ . Statistical analyses were performed using the Statistical Package for Social Sciences software (SPSS version 18.0).

## RESULTS

### Database of EUS images

Between March 2005 and December 2007, 153 patients with pancreatic cancer and 63 non-cancer patients with a normal pancreas (20 patients) or chronic pancreatitis (43 patients) were included in the analysis database. All EUS images of these patients were analyzed. The ROIs were drawn, and texture features were extracted and selected.

### Characteristics of the included patients

From April 2007 to March 2009, a total of 25 consecutive patients were enrolled. There were fourteen men and eleven women, with a median age of 67 years (range 54-80 years) and a median KPS score of 80 (range 60-90). Five patients were in stage III, and twenty were in stage IV. The average number of seeds (0.5 mCi per seed) implanted was 14.6 per patient (range 5-30 per patient). All patients tolerated the brachytherapy well without any complications throughout the study.

### Change of the fuzzy classification score

A total of 250 EUS images from the 25 patients were analyzed using the fuzzy classification method, and every patient was scored twice. There was a correlation between the change in the fuzzy classification score and overall survival ( $r = 0.616$ ,  $P = 0.001$ ), whereas no correlation was found to be significant between the change of tumor volume ( $P = 0.663$ ), CA199 level ( $P = 0.659$ ), and overall survival. There were 15 patients with a decrease in the fuzzy classification score after the brachytherapy, whereas the fuzzy classification score increased in other 10 patients (Table 1). There was a significant difference in the overall survival between the two groups (67 d *vs* 151 d,  $P = 0.001$ , Figure 1). There was no significant difference in the change of tumor volume ( $P = 0.345$ ) and CA199 level ( $P = 0.371$ ) between the two groups (Table 1).

**Table 1** The change in the fuzzy classification result and clinical parameters after brachytherapy in 25 unresectable pancreatic cancer patients

No.	FCS before brachytherapy	FCS after brachytherapy	FCS <sup>1</sup>	Tumor volume <sup>1</sup>	CA199 <sup>1,2</sup>	Survival time (d)
1	22.30	80.40	-260.50%	-93%	-53%	58
2	16.30	33.52	-105.60%	14%	69%	69
3	70.60	88.61	-25.50%	42%	75%	50
4	50.30	60.24	-19.80%	53%	0%	80
5	52.60	56.80	-8.00%	2%	0%	53
6	60.30	63.50	-5.30%	0%	8%	67
7	87.52	90.31	-3.20%	0%	66%	198
8	88.50	90.20	-1.90%	-320%	17%	54
9	83.10	83.68	-0.70%	0%	NA	103
10	90.80	91.20	-0.40%	12%	0%	125
11	88.20	87.20	1.10%	-15%	NA	200
12	90.20	88.93	1.40%	-171%	0%	143
13	91.30	88.70	2.80%	0%	0%	138
14	92.43	89.20	3.50%	48%	0%	221
15	95.10	88.20	7.30%	-1%	6%	312
16	89.21	80.12	10.20%	-104%	-30%	108
17	93.70	81.10	13.40%	71%	85%	61
18	87.52	75.21	14.10%	44%	-265%	182
19	92.20	61.23	33.60%	96%	0%	122
20	82.30	47.60	42.20%	78%	-3%	200
21	78.56	44.00	44.00%	-66%	-1%	150
22	75.90	35.62	53.10%	42%	13%	104
23	51.20	18.30	64.30%	40%	-358%	151
24	90.10	22.70	74.80%	61%	96%	194
25	89.30	18.64	79.10%	4%	95%	378
Groups						
Increase ( <i>n</i> = 10) (interquartile range)			-6.7% (43.9%)	1% (44%)	8% (68%)	67 (49) <sup>3</sup>
Decrease ( <i>n</i> = 15) (interquartile range)			14.1% (49.6%)	40% (76%)	0% (41%)	151 (78) <sup>3</sup>

<sup>1</sup>The parameter change was calculated by (pre-post)/pre; <sup>2</sup>When the levels of carbohydrate antigen 199 (CA199) were larger than 1000 μmol/L before and after treatment, 0% meant no improvement; <sup>3</sup>*P* = 0.001. FCS: Fuzzy classification score; NA: Not available.

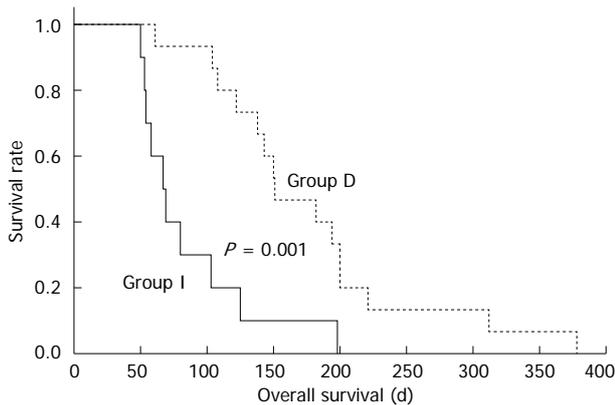
## DISCUSSION

The analysis of texture features is the core of DIP of digital images. Texture features are helpful for classifying lesions on sonography, and the potential of sonographic texture analysis to improve tumor diagnosis has already been demonstrated<sup>[23-27]</sup>. However, only a few reports exist about the application of DIP techniques to EUS. For the diagnosis of pancreatic cancer, research using DIP and pattern recognition remains rare. Two recent studies successfully used neural network analysis of EUS images to differentiate pancreatic cancer from chronic pancreatitis<sup>[1,3]</sup>. Das *et al.*<sup>[3]</sup> reported high sensitivity (93%) and specificity (92%), with excellent positive predictive values (87%) and negative predictive values (96%). An SVM model was evaluated as a potential method to differentiate between malignant and benign lesions with excellent accuracy rates<sup>[28]</sup>. Its performance characteristics in differentiating pancreatic cancer from benign lesions or normal tissue of the pancreas are closely rivaled by those of EUS-FNA.

In our study, the feature extraction and selection based on fuzzy classification was applied to EUS images of pancreatic cancer patients. All the work was carried out by the developed C++ program. According to the fuzzy algorithm, the classification result was not just “yes” or “no”, but a score from 0 to 100<sup>[2,13,29]</sup>. Compared with the SVM method<sup>[13]</sup>, the fuzzy classification method

proposed in our study could additionally give the precise numerical difference between a cancer case and a non-cancer case.

EUS-guided brachytherapy has been applied preliminarily in the study of advanced pancreatic cancer<sup>[30]</sup>. Two clinical series showed that pancreatic cancer could be treated safely with EUS-guided brachytherapy with pain control<sup>[31,32]</sup>. The number of patients enrolled in these two series was 22 and 100, respectively, with stage III or IV pancreatic cancer in a majority of cases. The estimated median overall survival in the two studies was 9.0 and 7.0 mo. The brachytherapy’s effect on overall survival was uncertain because of the lack of a control. Meanwhile, making prognosis judgments for these patients is still difficult. Given that brachytherapy aims to destroy the tumor closely, if it were effective, the EUS images of pancreatic cancer ought to change to be more similar to those from a normal pancreas, and the fuzzy classification score of EUS images after the brachytherapy ought to show a decrease. Thus, the change of the fuzzy classification score most likely reflected the treatment effect to some extent and the prognosis after brachytherapy. Our study results validated this hypothesis. First, the change of the fuzzy classification score was significantly correlated with overall survival, which meant the more the score decreased, the longer the patient survived. Second, 15 of 25 patients (60%) had a decreased fuzzy classification score after the



**Figure 1** Survival curve of two groups of patients with pancreatic cancer treated with endoscopic ultrasonography-guided interstitial brachytherapy. Group I ( $n = 10$ ): Patients whose fuzzy classification score increased after the brachytherapy. Group D ( $n = 15$ ): Patients whose fuzzy classification score decreased after the brachytherapy.

brachytherapy. The median overall survival was nearly 5 mo. As a control, the fuzzy classification results increased in 10 patients after treatment, and the median overall survival was only approximately 2 mo. The log-rank test indicated a significant difference between these two groups.

The tumor volume is an important candidate for making prognosis evaluations for pancreatic cancer. In our study setting, the metal package of radioactive seeds made it difficult to measure the tumor volume by computed tomography. Thus, the EUS scan was a more suitable and convenient way to measure the volume. Meanwhile, as a diagnostic marker, CA199 is also another candidate for prognosis evaluation<sup>[53]</sup>. However, our results found no association between the change of tumor or CA199 and the overall survival, which meant they were not a suitable prognosis marker in the patient population.

There were some limitations in our study. The new method can distinguish pancreatic cancer from chronic pancreatitis or a normal pancreas, but it cannot differentiate different cancer types. The probable approach to overcome this problem is to train multiple, 1-*vs*-all classifiers<sup>[19]</sup>. Furthermore, enlarging the sample size and selecting new effective features are future possibilities for further study to improve the practicability of the technique.

In conclusion, the fuzzy classification method to score texture features of pancreatic cancer in EUS images is feasible and can be used as an effective tool to judge the prognosis of patients with unresectable pancreatic cancer treated by interstitial brachytherapy.

## COMMENTS

### Background

The application of digital image processing (DIP) in endoscopic ultrasonography (EUS) images and other imaging scenarios has been proven to be a useful adjunct to endoscopic diagnoses and is often comparable with specialists' interpretations in different pathologic settings. The typical method of support vector machine, which is only able to provide a differential diagnosis for solid tumors

("yes" or "no"), cannot provide numerical data describing the texture parameters in the EUS image. Thus, authors applied a new DIP method based on fuzzy classification to quantify the images and supply more information about the status of pancreatic cancer.

### Research frontiers

The prognosis for pancreatic cancer is poor, and the effects of all currently available therapies are poor. EUS-guided brachytherapy has been applied preliminarily in the study of advanced pancreatic cancer as a potential new therapy. However, making prognosis judgments for these patients after brachytherapy is still difficult. EUS has become a useful tool to monitor cancer lesions. DIP of the change in EUS images after brachytherapy may be useful for making prognosis judgements.

### Innovations and breakthroughs

Making prognosis judgments for patients with unresectable pancreatic cancer after EUS-guided interstitial brachytherapy is difficult. Authors developed a new DIP method based on fuzzy classification to analyze EUS images of pancreatic cancer, and they validated its utility in making prognosis judgements.

### Applications

The new DIP method based on using fuzzy classification to analyze EUS images supplies more information than other DIP methods and has a significant potential to assist in clinical decision making in terms of diagnosis, prognosis, and diseases monitoring, especially for solid tumors.

### Terminology

Fuzzy classification is the process of grouping elements into a fuzzy set whose membership function is defined by the truth value of a fuzzy propositional function.

### Peer review

This study provided a new method to evaluate the effect of EUS-guided interstitial brachytherapy on unresectable pancreatic cancer. Through digital image processing of EUS images, the current study indicates that using the fuzzy classification method to score the texture features of pancreatic cancer in EUS images is useful for making prognosis judgments for patients with unresectable pancreatic cancer treated by interstitial brachytherapy.

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## Ectopic liver: Different manifestations, one solution

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### Abstract

Developmental abnormalities are rare in the liver. This study presents two case reports of ectopic liver. The first case was a 31-year-old male with clinical indication for laparoscopic appendectomy. Laparoscopy identified a perforated appendix and an unknown tumorous lesion in the ligamentum hepato umbilicalis. The patient underwent a laparoscopic appendectomy, intraoperative lavage of the peritoneal cavity, and extirpation of the lesion in the ligamentum hepato umbilicalis. Histopathological examination of the excised tumor revealed that it comprised liver tissue with fibrinous changes. The tumor was completely separate from the liver with no connection. It was classified as an ectopic liver. No further therapy was required. The second case was a 59-year-old male with a tumor on the upper pole of the spleen, incidentally diagnosed in an ultrasound examination. The biopsy raised suspicion of hepatocellular carcinoma. A positron emission tomography-computed

tomography examination revealed accumulation of F-18 fluorodeoxyglucose only in the tumor. The patient underwent a splenectomy with a resection and reconstruction of diaphragm. After the hepatocellular carcinoma was confirmed, adjuvant therapy (sorafenib) was initialized. The operations and postoperative recoveries were uncomplicated in both cases. Despite the low incidence of ectopic liver and rare complications, it is necessary to maintain awareness of this possibility. The potential malignancy risk for ectopic liver tissue is the basis for radical surgical removal. Therapy for hepatocellular carcinoma in an ectopic liver follows the same guidelines as those followed for treating the "mother" liver.

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**Key words:** Ectopic; Liver; Hepatocellular carcinoma; Diagnostic; Treatment

**Core tip:** Ectopic liver presents a rare clinical finding resulting from liver tissue migration to various organs during embryogenesis. Although the condition is typically asymptomatic, it can lead to different clinical manifestations such as intraabdominal bleeding or hepatocarcinogenesis. The potential malignancy risk is the basis for radical surgical removal; which represents the only correct solution. Therapy for hepatocellular carcinoma in an ectopic liver follows the same guidelines (National Comprehensive Cancer Network Guidelines) as those followed for treating the "mother" liver. Despite the low incidence of ectopic liver and rare complications, it is necessary to maintain an awareness of this possibility.

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## INTRODUCTION

The liver is the largest abdominal organ. It occupies a substantial portion of the upper abdominal cavity. Abnormalities in the position or number of liver parts are considered rare developmental anomalies. They are typically asymptomatic, and incidental detection, though extremely rare, may occur during an operation or autopsy. The incidence of ectopic liver is 0.24%-0.56%, according to data described in laparoscopic or autopsy studies<sup>[1-3]</sup>, but this estimate seems high. Most authors distinguish two types of ectopic liver. The first is an accessory liver lobe connected to the liver, and the second is a truly ectopic liver. Collan classified four types. The first is the ectopic liver, which is not connected to the mother liver, but is typically attached to the gallbladder or intra-abdominal ligaments. The second is a microscopic ectopic liver, which is occasionally found in the gallbladder wall. The third is a large accessory liver lobe, attached to the mother liver by a stalk (pedunculated liver). The fourth is a small, accessory liver lobe attached to the mother liver<sup>[4]</sup>. Here, we presented two manifestations of ectopic livers.

## CASE REPORT

### Case 1

A 31-year-old male patient was admitted with an 8-h history of pain in the right lower abdominal quadrant with a gradual onset. The patient reported nausea, but no vomiting, normal bowel function, and normal miction. He was subfebril, but no infection was observed. His medical history included pollinosis. He took no regular medication and had no previous surgeries.

The clinical examination showed right lower quadrant abdominal pain with tenderness. The bowel sounds were diminished. The patient was hemodynamically stable without any signs of sepsis (temperature 37.5 °C, noninvasive blood pressure 120/80 mmHg, heart rate 76 beats/min, respiratory rate 14 breaths/min). The white blood count was 14800 cells/mL and C-reactive protein was 12.3 mg/L. Other biochemical results were normal and the urinalysis revealed no pathological findings. An abdominal ultrasound showed a small amount of pericapsular fluid. No other abnormal findings were identified by ultrasound in other parts of the abdomen or pelvis.

The signs and symptoms suggested appendicitis; therefore, we performed an acute laparoscopic appendectomy. First, antibiotic therapy was introduced. Initially, the routine diagnostic laparoscopy revealed perforated appendicitis with circumscribed peritonitis. Incidentally, a small oval tumor (3 cm × 2 cm × 2 cm) was found in the ligamentum hepato umbilicalis next to the liver (Figure 1). No other pathological signs were observed. The appendectomy was performed, followed by an intraoperative lavage of the peritoneal cavity. The tumor was excised. The operation and the postoperative recovery were uncomplicated. The patient was discharged on the third postoperative day. The histology of the appendix revealed an ulcerophlegmonous appendix. The histopath-



Figure 1 Small oval tumor (3 cm × 2 cm × 2 cm) was found in the ligamentum hepatoumbilicalis next to the liver.

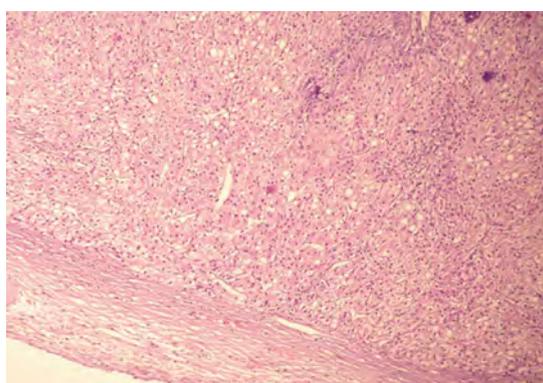


Figure 2 Histopathology of the tumor from the ligamentum hepatoumbilicalis (liver tissue with moderate steatosis and a thick fibrotic capsule).

ological examination of the tumor from the ligamentum hepato umbilicalis revealed liver tissue with moderate steatosis and a thick fibrotic capsule (Figure 2). The specimen examination showed that the tumor was completely separate, with no connection to the liver. It was classified as the first type of ectopic liver according to the Collan classification. No further therapy was required.

### Case 2

During an ultrasound examination, a 59-year-old male was incidentally diagnosed with a tumor (10 cm × 8 cm × 6 cm) on the upper pole of the spleen. The finding was confirmed with a computed tomography (CT) scan (Figure 3), and a biopsy was performed. A histological examination of the biopsy specimen, including immunohistochemistry, raised the suspicion of a metastatic hepatocellular carcinoma, but bile production was not caught and renal carcinoma could not be reliably ruled out. To detect the primary tumor location, we performed a positron emission tomography-computed tomography examination. This showed a localized accumulation of F-18 fluorodeoxyglucose only in the suspicious tumor. There was no other pathological finding in the abdominal or thoracic cavities. The medical history included toxic-nutritive hepatopathy (alcoholic liver disease) and chronic gastritis. The biochemical results were within normal

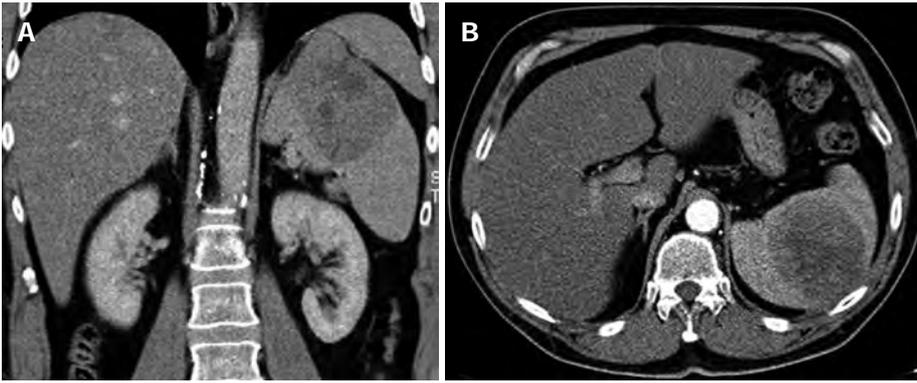


Figure 3 Tumor (10 cm × 8 cm × 6 cm) on the upper pole of the spleen. A: Computed tomography scan, arterial phase with a coronal reconstruction; B: Computed tomography scan, arterial phase - axial orientation.

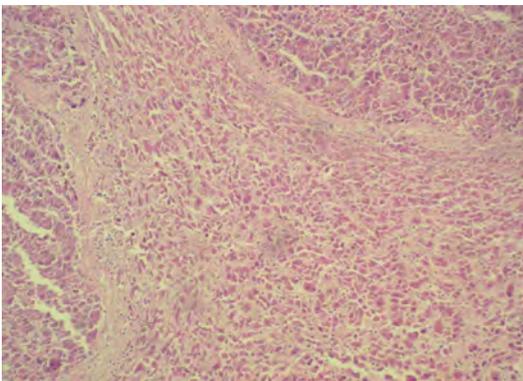


Figure 4 Histopathology of the hepatocellular carcinoma found in an ectopic liver in the spleen.

limits, and oncomarkers (carcinoembryonic antigen, alfa-fetoprotein, CA 19-9) were negative. A perioperative examination confirmed that the tumor was located on the upper pole of the spleen and was connected to diaphragm, but did not invade other surrounding tissues. It was classified as the first type of ectopic liver according to the Collan classification. No other pathology was found in the abdomen. A splenectomy was performed, with partial diaphragm resection and reconstruction. The postoperative recovery was uneventful. A definitive histological examination, including immunohistochemistry, confirmed a hepatocellular carcinoma (HCC) in the spleen tissue (Figure 4). Two small additional tumor sites (satellite tumors) were found in addition to the main lesion. Several investigations showed that the orthotopic liver tissue was negative for HCC, but a histological verification (a biopsy of the mother liver tissue) was not performed. Due to the high risk of tumor recurrence (additional tumor sites were found), we initialized a targeted adjuvant therapy with sorafenib. The American Association for the Study of Liver Diseases (AASLD) practice guidelines on the management of hepatocellular carcinoma do not recommend the routine use of adjuvant therapy with sorafenib (recurrence rates reduction was not reliably proven)<sup>[5]</sup>, nevertheless, there are data available that indicate that sorafenib was effective for treating patients with advanced HCC<sup>[6,7]</sup>.

## DISCUSSION

In development, the hepatic diverticulum comprises the liver and biliary tree, and it appears late in the third week or early in the fourth week of gestation. The foregut endoderm of the hepatic diverticulum develops into the liver parenchyma (hepatocytes) and the epithelial lining of the biliary tract. The hepatic diverticulum divides to form a small ventral portion, the future gall bladder, and a larger cranial portion, the liver primordium. Developmental errors are relatively rare in the liver. Other errors in foregut development are more frequently observed, like errors in pancreas or duodenum formation<sup>[8]</sup>. Liver tissue can migrate to various organs during embryogenesis. Sites of ectopic liver include the gallbladder, spleen, retroperitoneum, pancreas, adrenal gland, portal vein, diaphragm, thorax, gastric serosa, testes, and umbilical vein<sup>[9]</sup>. Most authors distinguish ectopic and accessory liver formations, based on whether there is a connection to the mother liver. The Collan Classification mentioned above is not widely used. In many cases, it is difficult to make a clear distinction between ectopic liver and accessory liver. The precise incidence of ectopic liver or accessory liver is unknown. Examination of several studies indicated that the incidence is approximately 0.24%-0.56%. Watanabe's series of 1060 patients revealed an incidence of 0.47% for ectopic liver and 0.09% for accessory liver. These numbers could be over-estimated, because histological verification was not performed in all cases. Ectopic and accessory liver are typically asymptomatic, but occasionally they cause unexpected problems, like intra-abdominal bleeding or hepatocarcinogenesis. The first clinical sign of ectopic or accessory liver could be an acute complication that leads to acute surgery and diagnosis of ectopic liver. Various clinical symptoms, like recurrent abdominal pain and impaired liver function could be caused by ectopic or accessory liver, but in the majority of cases, an ectopic/accessory liver remains undetected.

In some cases, the ectopic or accessory liver may undergo torsion, infarction, rupture, or other disorders. Torsion and subsequent infarction of an accessory liver lobe has been described in children and adults<sup>[10-14]</sup>. Ladurner

presented a very interesting case of a patient with hepatic ischemia caused by complete vascular occlusion due to a twisted accessory liver lobe. In that case, the accessory liver lobe produced serious, life-threatening problems, and an orthotopic liver transplantation was performed<sup>[15]</sup>. Ito reported a small omphalocele that involved an accessory liver lobe embedded in the cranial portion of the amniotic sac. In that case, the pedicle of liver tissue was markedly elongated<sup>[16]</sup>.

An ectopic or accessory liver can lead to benign or malignant diseases. Benign cases in the literature report hemangiomas, adenomas, or focal nodular hyperplasia associated with an ectopic or accessory liver<sup>[17-19]</sup>. Benign lesions seem to be less frequent; however, the higher frequency of malignancies could be based on the fact that many benign lesions remain undiagnosed because they are asymptomatic. Moreover, due to their abnormal locations, asymptomatic lesions may be misdiagnosed in the absence of histology.

The ectopic liver has been associated with malignancies more often than with benign lesions. Many authors have pointed out that ectopic liver tissue is more predisposed to malignancy than normal liver tissue. Ectopic livers have completely functional architecture, but may be metabolically handicapped; this may facilitate carcinogenesis. Ectopic liver tissue also has increased neoplastic potential compared to orthotopic liver tissue. This may have given rise to the hypothesis that ectopic livers are particularly predisposed to the development of hepatocellular cancer. A high incidence of hepatocellular cancer in ectopic livers was described in Japan<sup>[20]</sup>. In most cases, a malignant tumor was found in the ectopic liver, but not in the mother liver. Ectopic or accessory livers with cancer may be amenable to surgical resection. Many case reports have described surgical treatments. Some authors suggested that the outcome after resection to remove hepatocellular cancer was superior when it involved an ectopic or accessory liver, compared to when it involved the mother liver. However, long-term follow-up data are poor.

Many anatomical locations have been described for ectopic livers with cancer<sup>[20-23]</sup>. The favorable outcome after resection of ectopic livers could depend on the specific anatomical location<sup>[24,25]</sup>. Shigemori described a case of ectopic hepatocellular carcinoma in the jejunum<sup>[26]</sup>. Cardona *et al.*<sup>[27]</sup> reported a case of a primary, well-differentiated hepatocellular carcinoma arising from ectopic liver tissue in the pancreas. Leone presented interesting data regarding three cases of hepatocellular carcinomas that arose in ectopic livers. The clinical presentations were very interesting; one patient reported dull epigastric pain; the second reported abrupt onset with signs and symptoms of acute abdomen caused by intra-abdominal bleeding; and the third presented with an unexplained, progressive increase in alpha-fetoprotein serum levels<sup>[28]</sup>. Seo *et al.*<sup>[29]</sup> reported a case of hepatocellular carcinoma that arose from hepatic parenchyma located in the left subphrenic space in the upper portion of the gastrosplenic

ligament. The preoperative diagnosis was a nonspecific stomach mass, with suspicion of gastrointestinal stromal tumor. An operation was performed laparoscopically. Takavasu reported another case with high serum alpha-fetoprotein combined with a suspicion of a submucosal stomach tumor<sup>[30]</sup>. The two latter cases diagnosed the ectopic liver postoperatively, after histological examination.

It is important to consider an ectopic/accessory liver when evaluating perihepatic lesions. It is common to misdiagnose an ectopic liver as a malignant tumor. Stat-taus suggested that the diagnosis should be based on a biopsy of ectopic liver or a NMR with liver specific contrast<sup>[31]</sup>. However, all investigative imaging methods are limited for diagnosing an ectopic/accessory liver, due to its limited volume. Despite the low incidence and rare complications of ectopic/accessory liver, it is necessary to maintain an awareness of this possibility. Because this entity presents with a broad spectrum of clinical symptomatology, it is rarely diagnosed; thus, most discoveries of ectopic and accessory liver are incidental. An elevation in serum alpha-fetoprotein and lack of focus in liver CT image may be the first signs of malignant transformation in an ectopic liver. The suspicion of hepatocellular carcinoma in an ectopic liver is substantial reason for radical surgical removal of an ectopic liver found incidentally. When hepatocellular carcinoma is definitely, histologically confirmed in an ectopic liver, it should be treated with the same approaches used for treating carcinoma in the mother liver (National Comprehensive Cancer Network Guidelines).

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## A case of a duodenal duplication cyst presenting as melena

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**Key words:** Gastrointestinal hemorrhage; Duodenum; Duplication

**Core tip:** Duodenal duplication cysts are rare congenital anomalies that have been seldom reported in adults. Most cases of duodenal duplication have been associated with pancreatitis or jaundice and few have been reported as a cause of gastrointestinal hemorrhage. We submit a case of duodenal duplication cyst causing gastrointestinal hemorrhage. In rare cases, duodenal duplication cysts may cause gastrointestinal bleeding and must be included in the differential diagnosis.

Ko SY, Ko SH, Ha S, Kim MS, Shin HM, Baeg MK. A case of a duodenal duplication cyst presenting as melena. *World J Gastroenterol* 2013; 19(38): 6490-6493 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6490.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6490>

### Abstract

Duodenal duplication cysts are benign rare congenital anomalies reported mainly in the pediatric population, but seldom in adults. Symptoms depend on the type and location and can present as abdominal pain, distension, dysphagia or dyspepsia. They have been reported to be responsible for duodenal obstruction, pancreatitis and, in rare cases, gastrointestinal bleeding. We present a case of a duodenal duplication cyst in a 43-year-old man presenting as melena. Initial gastroduodenoscopy and colonoscopy did not reveal any bleeding focus. However, the patient began passing melena after 3 d, with an acute decrease in hemoglobin levels. Subsequent studies revealed a duplication cyst in the second portion of the duodenum which was surgically resected. Histology revealed a duodenal duplication cyst consisting of intestinal mucosa. There was no further bleeding and the patient recovered completely. In rare cases, duodenal duplication cysts might cause gastrointestinal bleeding and should be included in the differential diagnosis.

### INTRODUCTION

Duplication cysts are rare congenital anomalies of the gastrointestinal (GI) tract. Duodenal duplication cysts are extremely rare, representing only 2%-12% of GI tract duplications<sup>[1]</sup>. Most duplication cysts are detected in children and fewer than 30% of all duplications are diagnosed in adults<sup>[2]</sup>. They are difficult to diagnose, as the presenting symptoms are nonspecific and are closely related to the type, size and location of the lesion<sup>[1]</sup>. We report a rare case of duodenal duplication with duodenoduodenal intussusception presenting as GI bleeding.

### CASE REPORT

A 43-year-old man was admitted to the emergency department complaining of melena. He had complained of recurrent burning, nonradiating upper abdominal pain,

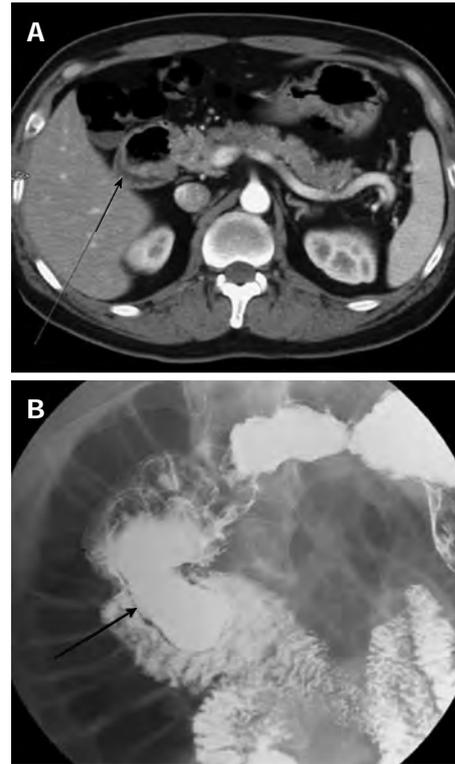
usually lasting for several minutes, irregular in nature and relieved by taking food since childhood. Ten years previously, he had been diagnosed with a gastric ulcer and had taken medications for 1 year.

His vital signs upon admission were a blood pressure of 120/80 mmHg and a heart rate of 105 beats/min. Initial laboratory studies revealed a hemoglobin level of 9.7 g/dL (normal range, 14-18 g/dL), hematocrit 28.8% (normal range, 42.0%-52.0%) and mean red corpuscular volume 88.4 fL (normal range, 80-94 fL). A nasogastric tube was inserted, but no blood was aspirated.

An initial gastroduodenoscopy showed no evidence of active bleeding or obvious focus. A colonoscopic examination was unremarkable, except for the presence of internal hemorrhoids. After a blood transfusion of 220 mL packed red blood cells, his hemoglobin level rose to 10.3 g/dL and there was no clinical evidence of further bleeding.

On the third day of hospitalization, the patient passed about 200 mL of melena and his hemoglobin levels fell to 7.6 g/dL. Gastroduodenoscopy revealed fresh blood oozing without a definite bleeding focus in the second portion of the duodenum. As there was no obvious bleeding focus, a high-resolution computed tomography (CT) scan of the abdomen was done. It revealed a circumferential cystic lesion in the proximal duodenum arising from a duodenoduodenal intussusception (Figure 1). As the patient did not have any symptoms except for melena and physical examinations did not reveal any palpable sausage-shaped abdominal mass suggestive of intussusception, an upper GI series was done. This demonstrated a large elongated sac-like 10 cm long mass in the second and proximal third portion of the duodenum (Figure 2). The junction of the first and second portions of the duodenum, where the mass began, was narrowed locally and the second portion of the duodenum was enlarged. This feature suggested a large elongated communicating duplication cyst (about 10 cm long) in the second and proximal third portion of the duodenum with duodenoduodenal intussusception.

As rare cases of GI cysts with malignancies have been reported in the literature, bleeding from a cancer could not be ruled out. Because of the recurrent bleeding symptoms and the possibility of a cancer being present, we chose surgical resection. This revealed a mass attached to the second and third portion of the duodenum. After duodenotomy, the cyst was opened and blood clots were found in its cavity. Subtotal resection of the cyst was done followed by primary closure of the duodenum. A macroscopic examination showed an irregular, flat mass measuring 4 cm × 4 cm × 0.5 cm, covered with intestinal mucosa and with hemorrhagic contents (Figure 3). Microscopy revealed showed the cyst wall to be composed of two mucosal layers sharing a common muscle layer (Figure 3). The patient had an uneventful recovery and was discharged 12 d later. Follow-up visits at our outpatient clinic have revealed no sign of further GI bleeding.



**Figure 1** Computed tomography image. A: A high resolution computed tomography image of the abdomen reveals a circumferential cystic lesion (arrow) in the vicinity of the second part of the duodenum; B: Upper gastrointestinal series showing an large elongated sac-like mass (about 10 cm in length, arrow) arising from the second and proximal third portion of the duodenum.

## DISCUSSION

GI duplication cysts are rare congenital anomalies formed during the embryonic development of the alimentary tract. They are defined by a smooth muscle coat, an intimate attachment to the native GI tract and a GI mucosal lining<sup>[3]</sup>. They can occur anywhere along the GI tract, with varying types, shapes and sizes. GI duplication cysts are most commonly found in the distal ileum, followed by the esophagus, colon and jejunum<sup>[4]</sup>.

Duodenal duplication cysts are among the rarest of all intestinal duplications. Most are located in the second or third portion of the duodenum. The cysts are usually filled with clear fluid, but might contain gallstones, bile or pancreatic fluid depending on communication with the biliary or pancreatic systems<sup>[5]</sup>. Most are diagnosed in childhood, with diverse, nonspecific clinical presentations<sup>[6]</sup>. The most common symptoms reported are abdominal pain and nausea/vomiting. The most common complication is pancreatitis, reported in up to 53% of patients. Other manifestations such as intussusceptions, infection or weight loss have also been reported<sup>[1]</sup>. Though rare, GI bleeding can result from peptic ulceration of the ectopic gastric mucosa within the cyst and be a cause of unexplained GI bleeding<sup>[3]</sup>. The bleeding can be painless, brisk and life threatening, as shown in our patient. The bleeding in our case was made more interesting by the

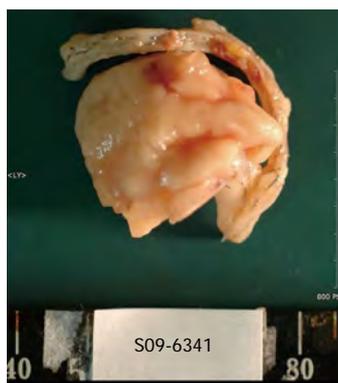


Figure 2 Gross specimen of the excised duodenal duplication cyst.

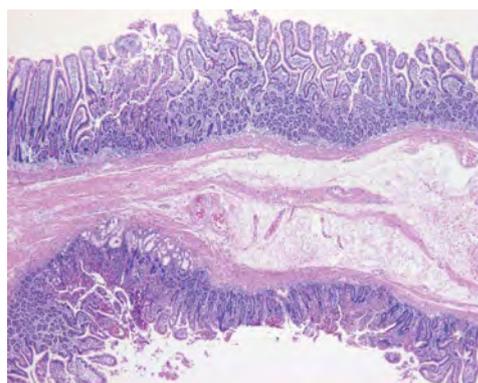


Figure 3 Histology of the duplication cyst showing two mucosal layers sharing a submucosal layer with muscular layer. Some of the mucosa are comprised of duodenal mucosa and the majority are jejunal mucosa (hematoxylin and eosin stain,  $\times 40$ ).

fact that histology did not reveal any ectopic gastric mucosa within the cyst, contrary to previous reports<sup>[1,7,8]</sup>.

The diagnosis of duodenal duplication cysts is difficult. The site of origin, such as an intraluminal or intramural position, and the presence or absence of luminal communication has to be taken into account. If the cyst is intraluminal, differentiation must be made between a pedunculated neoplastic lesion or a duodenal diverticulum. In the case of an intramural mass, a distended duodenal duplication has to be differentiated from other cystic masses belonging to the duodenal-choledochal-pancreatic area such as cystic dystrophy of the duodenal wall, choledochal cyst, pseudocysts, cystic tumors of the pancreas, cystic lymphangiomas, or mesenteric cysts. An empty duodenal duplication shows a solid structure and has to be differentiated from neoplastic or inflammatory duodenal or pancreatic lesions<sup>[9]</sup>.

The preoperative diagnosis of duodenal duplications is often inaccurate. Diagnosis is usually done using imaging modalities such as ultrasonography or CT scans. On ultrasonography, duodenal duplication is seen as having an echogenic inner mucosa surrounded by a hypoechoic outer muscular layer<sup>[4]</sup>. CT scans often reveal a cystic mass associated with the alimentary tract and is more useful in demonstrating the precise anatomical relationship between the cyst and surrounding structures<sup>[4]</sup>. Magnetic resonance images as well as endoscopic ultrasonography allow us to suspect duplications and are useful in evaluating upper GI tract masses<sup>[3,9]</sup>. On contrast CT series of the GI, duodenal duplication cysts can present as smooth submucosal or extrinsic masses, or as oval filling defects in the duodenum, as can be seen in our case. In case of bleeding, the most sensitive tool is gastroduodenoscopy, which can locate the bleeding duplication in the duodenum<sup>[8]</sup>. However, all modalities allow us only to suspect the presence of an abnormal lesion, and diagnostic confirmation is possible only after resection.

Treatment of duodenal duplication has classically involved surgical resection. However, cases of endoscopic treatment have been reported, especially in cases where the duplication is in close proximity to the adjacent structures such as the major duodenal papillae<sup>[10]</sup>. In our case,

endoscopic treatment was not possible because the duplication was not visible by endoscopy, leading to the need for surgical excision. Treatment of asymptomatic cases remains controversial. However, as neoplasms have been reported within duodenal duplication cysts, surgical resection must be considered<sup>[1]</sup>.

In conclusion, duodenal duplication cysts are rare congenital anomalies that are seldom reported in adults. Clinical presentations are diverse, but there are a few reports of GI bleeding. Here we report a case of a duodenal duplication cyst with overt GI bleeding and suggest that such cysts should be considered in cases of undiagnosed GI bleeding.

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**E-Editor** Zhang DN



## Simultaneous intrahepatic and subgaleal hemorrhage in antiphospholipid syndrome following anticoagulation therapy

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### Abstract

Warfarin is a widely used anticoagulant. Interindividual differences in drug response, a narrow therapeutic range and the risk of bleeding render warfarin difficult to use clinically. An 18-year-old woman with antiphospholipid syndrome received long-term warfarin therapy for a recurrent deep vein thrombosis. Six years later, she developed right flank pain. We diagnosed intrahepatic and subgaleal hemorrhages secondary to anticoagulation therapy. After stopping oral anticoagulation, a follow-up computed tomography showed improvement in the hemorrhage. After restarting warfarin because of a recurrent thrombosis, the intrahepatic hemorrhage recurred. We decided to start clopidogrel and hydroxychloroquine instead of warfarin. The patient has not developed further recurrent thrombotic or bleeding

episodes. Intrahepatic hemorrhage is a very rare complication of warfarin, and our patient experienced intrahepatic and subgaleal hemorrhage although she did not have any risk factors for bleeding or instability of the international normalized ratio control.

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**Key words:** Warfarin; Liver; Subgaleal; Hemorrhage; Antiphospholipid syndrome

**Core tip:** An 18-year-old woman with antiphospholipid syndrome received long-term warfarin therapy for a recurrent deep vein thrombosis. Six years later, she was diagnosed with intrahepatic and subgaleal hemorrhage and received clopidogrel and hydroxychloroquine in place of warfarin. She has not developed further recurrent thrombotic or bleeding episodes. Intrahepatic hemorrhage is a very rare complication of warfarin, and our patient experienced intrahepatic and subgaleal hemorrhages even though she did not have any risk factors for bleeding or an elevated international normalized ratio.

Park IC, Baek YH, Han SY, Lee SW, Chung WT, Lee SW, Kang SH, Cho DS. Simultaneous intrahepatic and subgaleal hemorrhage in antiphospholipid syndrome following anticoagulation therapy. *World J Gastroenterol* 2013; 19(38): 6494-6499 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i38/6494.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i38.6494>

### INTRODUCTION

Antiphospholipid syndrome (APS) is characterized by

venous or arterial thrombosis or pregnancy morbidity, in the presence of antiphospholipid antibodies (aPL). A venous thrombosis is the most common presenting complication of APS<sup>[1]</sup>. Long-term anticoagulation therapy is recommended for the secondary prevention of thromboembolism.

Warfarin is a widely used anticoagulant prescribed for patients with venous thrombosis, pulmonary embolism, chronic atrial fibrillation, and prosthetic heart valves. Interindividual differences in drug responses, a narrow therapeutic range and the risk of bleeding render warfarin difficult to use clinically. During treatment with oral anticoagulants, the risk of severe bleeding has been estimated to be 0.6%-1.0% per treatment year<sup>[2,3]</sup>. In a retrospective study of hemorrhagic complications in 184 patients, gastrointestinal bleeding was the most common complication, and liver or splenic hemorrhage was identified in only 3 patients<sup>[4]</sup>. Only a few cases of liver hematomas have been reported in the literature following anticoagulant therapy<sup>[5]</sup>. We report a woman with primary APS who presented with a simultaneous intraparenchymal hemorrhagic complication in the liver and a subgaleal hematoma with anticoagulation treatment, without any risk factors of bleeding or an elevated international normalized ratio (INR). This type of condition has not been previously described.

## CASE REPORT

An 18-year-old woman visited Dong-A University Hospital with swelling and tenderness of the left lower leg in March 2004. She had no past history of serious illness. Doppler ultrasonography (US) of her lower legs showed deep vein thromboses (DVT) in the left popliteal vein and the right common femoral vein. She received anticoagulation treatment with warfarin for 2 years. After 2 years, a follow-up US showed nearly complete improvement of the DVT, and anticoagulation therapy was stopped.

She returned four months later, in November 2006, with edema and tenderness of the right lower leg. US of her lower legs showed a chronic DVT in the right popliteal vein. We reexamined her for other conditions leading to a hypercoagulable state. The clinical lab values were as follows: white blood cell count (WBC) 4570/mm<sup>3</sup>, segmental neutrophils 29%, hemoglobin (Hb) 11.9 g/dL, platelets (PLT) 91000/mm<sup>3</sup>, prothrombin time (PT) 13.9 s, INR 1.23 and activated partial thromboplastin time (aPTT) 31.3 s. Tests for antinuclear antibodies were positive (at 1:40 dilution), and the particular types of immunofluorescence pattern were negative. An anti-double-stranded DNA antibody enzyme-linked immunoabsorbent assay test was moderately positive (326.7 WHO unit/mL). Anti-Smith antibody and anti-ribonucleoprotein antibody test were negative. Immunoglobulin (Ig)G anti- $\beta_2$ -glycoprotein I (anti- $\beta_2$  GPI) was positive (98.8 GPL), and IgM anti- $\beta_2$ -GPI was positive (95.5 GPL). IgG anticardiolipin antibodies (aCL) were highly positive (95.6 GPL),

and IgM aCL was positive (37.9 MPL). Lupus anticoagulant testing (diluted russell viper venom test, DRVVT) was positive (185.5 s). The patient was diagnosed with primary APS according to the revised Sapporo criteria for APS diagnosis<sup>[6,7]</sup>, and oral anticoagulation treatment was initiated.

After forty months of anticoagulation therapy, the patient was admitted in April 2010 with epigastric pain, headache, fever and chills. On physical examination, she had epigastric tenderness and body temperature of 38.2 °C. The laboratory findings were: WBC 17111/mm<sup>3</sup>, segmental neutrophils 82.9%, Hb 9.7 g/dL, PLT 136000/mm<sup>3</sup>, C-reactive protein (CRP) 8.38 mg/dL, alkaline phosphatase 211 I/U, PT INR 1.82 and aPTT 31.4 s. An abdominal computed tomography (CT) showed multiple low attenuated lesions without peripheral enhancement and perilesional edema of the liver on contrast-enhanced image (Figure 1). A brain magnetic resonance imaging (MRI) showed a crescentic high signal in the posterior parietal scalp on the T2 weighted image, a low signal on the T1 weighted image and mild enhancement on the contrast-enhanced scan images (Figure 2). We suspected intraparenchymal hemorrhage in the liver and a subgaleal hematoma in the posterior parietal scalp because of bleeding complications from anticoagulation. We stopped the warfarin treatment and administered antibiotics because there was the potential for an infected intrahepatic hemorrhage. After 2 wk of supportive care, a follow-up US on day 16 showed resolution of a previous hepatic lesion. We decided to restart warfarin because of her recurrent thrombotic episodes.

She was readmitted in June 2010 with right flank pain. On physical examination, she had abdominal tenderness in the right upper quadrant and her body temperature was normal. The WBC was 13250/mm<sup>3</sup>, the segmental neutrophils were 80.8%, the Hb was 10.2 g/dL and the PLT were 88000/mm<sup>3</sup>. The CRP was 7.93 mg/dL. The PT INR was 1.53, and the aPTT was 29.8 s. Liver and kidney function tests were unremarkable. An abdominal CT showed a low attenuated lesion in the S2/4 and S5 segments of the liver on the contrast-enhanced image (Figure 3A and B). We diagnosed the patient with intraparenchymal hemorrhage of the liver from the anticoagulation therapy and stopped warfarin treatment. Follow-up CT scanning on day 17 showed nearly complete improvement of intraparenchymal hemorrhage in the S2/4 and S5 segments of the liver (Figure 3C and D). After 1 mo, a follow-up US showed the previous intrahepatic lesions were completely resolved. We decided to initiate clopidogrel and hydroxychloroquine instead of warfarin. Subsequently, the patient has not developed recurrent thrombotic episodes or bleeding complications.

## DISCUSSION

APS is characterized by the occurrence of venous or arterial thromboses or by specific pregnancy morbidity in the presence of laboratory evidence of aPL. According

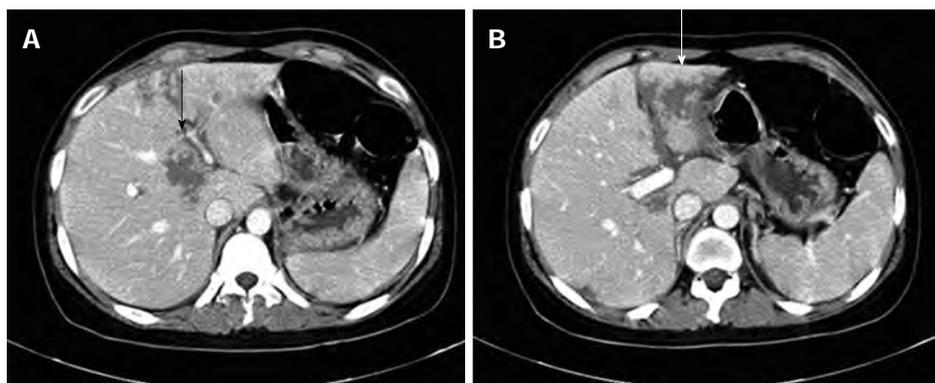


Figure 1 Intraparenchymal hemorrhage in the liver (arrows). An abdominal computed tomography showed multiple low attenuated lesions without peripheral enhancement (A) and perilesional edema of the liver on the contrast-enhanced image (B).

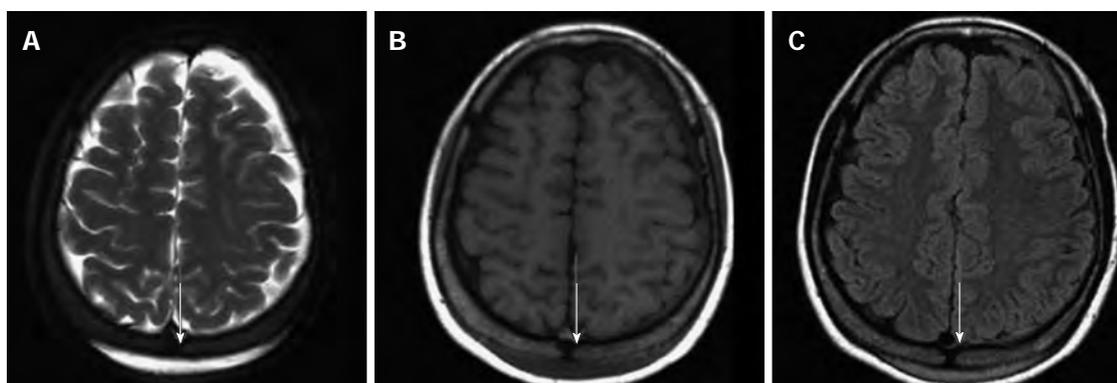


Figure 2 Subgaleal hematoma at the posterior parietal scalp (arrows). A brain magnetic resonance imaging showed a crescentic high signal in the posterior parietal scalp on the T2 weighted image (A), a low signal on the T1 weighted image (B) and mild enhancement on the contrast-enhanced image (C).

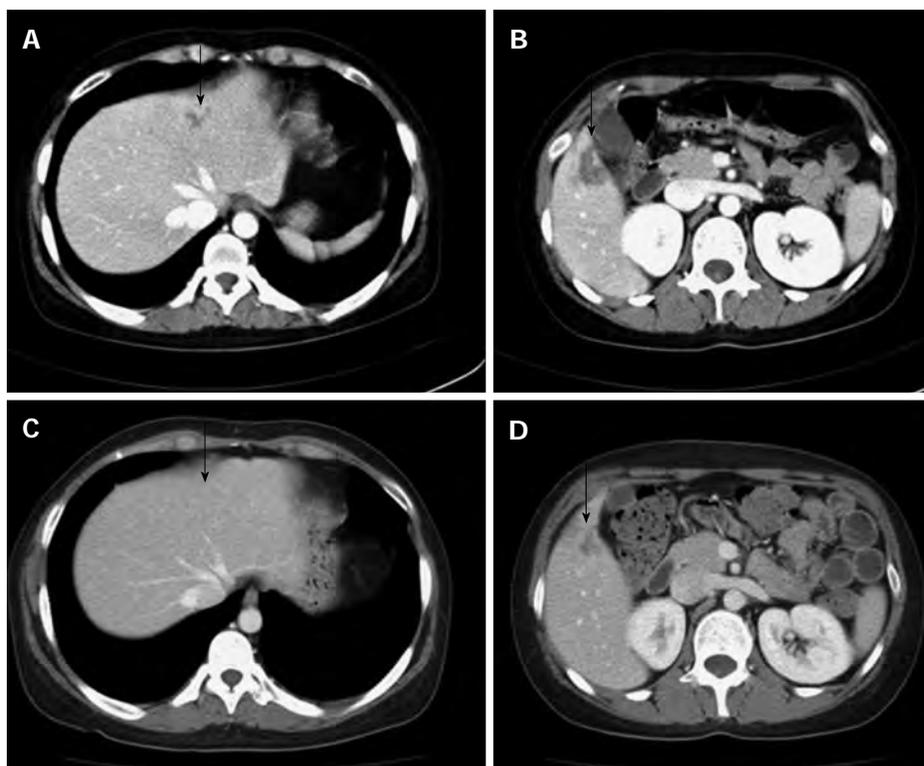


Figure 3 Recurrent and nearly complete resolution intraparenchymal hemorrhage in the liver (arrows). A, B: An abdominal computed tomography showed a low attenuated lesion (arrow) in the S2/4 (A) and S5 segments (B) of the liver on the contrast-enhanced image; C, D: An abdominal computed tomography showed resolution of the parenchymal hemorrhage in S2/4 (C) and the healing process in S5 (D) of the liver.

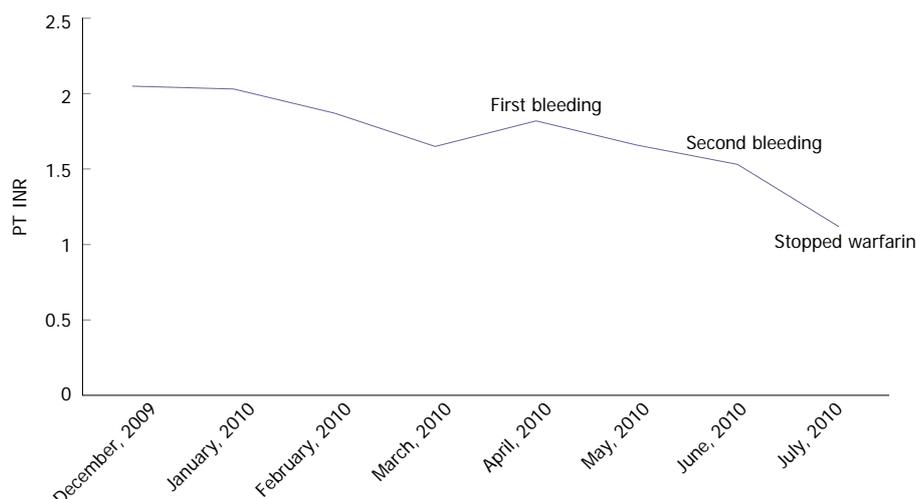


Figure 4 Extended follow-up data for the prothrombin time international normalized ratio. PT INR: Prothrombin time international normalized ratio.

to the revised Sapporo criteria<sup>[6,7]</sup>, definite APS is considered if at least one of the clinical criteria and at least one of the laboratory criteria are satisfied. APS occurs either as a primary or secondary condition in the setting of an underlying disease, usually systemic lupus erythematosus. In the largest prospective cohort study of patients with APS, venous thromboembolism was the most common presenting complication, including DVT (31.7%), pulmonary embolism (9.0%), and superficial thrombophlebitis (9.1%)<sup>[11]</sup>. Warfarin is the standard of care for the chronic management of patients with APS who are not pregnant.

Warfarin is an oral anticoagulant used in a variety of clinical settings. The anticoagulant effect of warfarin is mediated through inhibition of the vitamin K-dependent gamma-carboxylation of coagulation factors II, VII, and X. Warfarin is among the top 10 drugs with the largest number of serious adverse event reports submitted to the United States Food and Drug Administration, because it has a high incidence of adverse effects as well as variable interactions with diet or medications through a mechanism of altered platelet function, altered vitamin K synthesis in the gastrointestinal tract and interference with vitamin K metabolism<sup>[8-11]</sup>. In a prospective observational study, life-threatening bleeding occurred in 32 of 1999 patients. The gastrointestinal tract was the most common site, with bleeding occurring in 21 (66%) of the 32 patients<sup>[12]</sup>. In another study of hemorrhagic complications in 184 patients, gastrointestinal bleeding was the most common complication and unusual hemoperitoneum and/or soft tissue bleeding were reported<sup>[4]</sup>. Liver hemorrhage following anticoagulation therapy has been rarely reported in the literature<sup>[5,13-15]</sup>. It is sometimes difficult to distinguish hepatic hemorrhage from a liver abscess. Liver abscesses appear as low attenuated lesions with peripheral enhancement or perilesional edema<sup>[16,17]</sup>. We diagnosed a patient with an intrahepatic hemorrhage because these characteristics were not observed in our patient. We discussed the possibility of a microaneurysm in the liver and brain with radiology specialists who said that it was very unlikely.

There are many risk factors associated with bleeding complications following the use of vitamin K antagonist. These risk factors have been associated with a significantly increased risk of bleeding in one or more multivariate analyses: old age, diabetes mellitus, the presence of malignancy, hypertension, acute or chronic alcoholism, liver disease, severe chronic kidney disease, elevated creatinine, anemia, the presence of bleeding lesions or episodes, a bleeding disorder, concomitant use of aspirin, nonsteroidal anti-inflammatory drugs, antiplatelet agents, antibiotics, amiodarone, statins or fibrates, and instability of the INR (INR > 3.0, pre-treatment INR > 1.2)<sup>[18,19]</sup>.

In previously reported spontaneous liver hemorrhages, Erichsen *et al.*<sup>[14]</sup> addressed the severe drug interaction between warfarin and trimethoprim-sulfamethoxazole. Dizadji *et al.*<sup>[13]</sup> and Roberts *et al.*<sup>[15]</sup> showed the two risk factors of old age and high INR at the time of admission. Behranwala *et al.*<sup>[5]</sup> suggested multiple risk factors, including; old age, hypertension, instability of INR control, and drug interactions with statins and omeprazole. Our patient had a simultaneous intraparenchymal hemorrhage in the liver and subgaleal hematoma despite the absence of risk factors for bleeding and a therapeutic range of warfarin with a PT INR of 1.53 (Figure 4). This type of case has not been previously reported.

One study suggested risk factors that could be used in estimating the probability of major bleeding in outpatients treated with warfarin. The risk factors include the following; age  $\geq$  65 years, history of stroke, history of gastrointestinal bleeding, and one or more the following (recent myocardial infarction, hematocrit < 30%, serum creatinine > 1.5 mg/dL, and diabetes mellitus<sup>[20]</sup>). The cumulative incidence of major bleeding at 48 mo in the low (no risk factor), intermediate (1 to 2), and high (3 or more) risk groups was 3%, 12% and 53% respectively. Our patient belonged to the low risk group with a cumulative incidence of only 3%. Some recent reports showed that APS could be correlated to a transient hemorrhagic event without anticoagulation therapy<sup>[21,22]</sup>. Lupus anticoagulant-hypoprothrombinemia syndrome is a rare clinical

entity that can occur in association with systemic lupus erythematosus. It is characterized by prolongation of the coagulation test that is not corrected by normal fresh plasma because of non-neutralizing antibodies against Factor II. Our patient did not have this abnormal laboratory finding.

There are very few randomized studies comparing the treatment options for patients with an elevated INR and/or bleeding complications following the use of warfarin. If significant or life-threatening bleeding occurs, rapid reversal of excessive anticoagulation should be undertaken at any degree of anticoagulation<sup>[23-26]</sup>. Warfarin should be stopped, and 10 mg of vitamin K should be administered by a slow intravenous infusion, supplemented by fresh frozen plasma for less urgent situations. For more urgent situations, recombinant human factor VIIa or prothrombin complex concentrate may be used. If needed, angiography may help with embolization in cases of severe major bleeding. In the majority of cases, bleeding is improved by stopping warfarin and conservative care. Restarting of warfarin is not recommended, and changing the drug used for maintaining long-term anticoagulation is suggested<sup>[5]</sup>.

In studies of the bleeding risk during antithrombotic therapy, the odds ratio is generally lower for clopidogrel than warfarin, and we decided to start clopidogrel instead of warfarin<sup>[27,28]</sup>. We added hydroxychloroquine because limited data showed that it might be useful for thrombosis in patients with APS although there were no randomized controlled trials<sup>[29,30]</sup>. After starting clopidogrel and hydroxychloroquine, our patients did not experience additional bleeding complications or recurrent thromboses.

Our patient had a simultaneous intrahepatic hemorrhage and a subgaleal hematoma despite the absence of risk factors and a therapeutic range of warfarin. This is the first case with simultaneous bleeding in the liver and brain in an APS patient treated with warfarin.

Patients with intrahepatic bleeding might experience right upper quadrant or epigastric pain and pain that radiates into the shoulder or flank. Fever might develop when an infection accompanies the intrahepatic hemorrhage. If a patient experience pain and/or fever during anticoagulation therapy, we should consider the possibility of abdominal bleeding such as an intrahepatic hemorrhage in any patient receiving oral anticoagulants, even though this circumstance remains unlikely. CT or US should be adopted for early diagnosis.

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## Ileal duplication mimicking intestinal intussusception: A congenital condition rarely reported in adult

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**Key words:** Ileal duplication cyst; Adulthood; Computed tomography enterography; Tc-99m pertechnetate scintigraphy

**Core tip:** We reported a case of duplication of the alimentary tract involving the ileum, which mimicked intestinal intussusception, Meckel's diverticulum (MD) and Crohn's disease, in a young man. Computed tomography enterography identified the intussusception in the right lower quadrant, and the disease was positive on Tc-99m pertechnetate scintigraphy. Exploratory laparotomy and surgical pathology showed ileal duplication cyst with complicating ectopic gastric mucosa. Differential diagnosis of ileal duplication cyst was made, especially from MD.

### Abstract

Intestinal duplication is an uncommon congenital condition in young adults. A 25-year-old man complained of chronic, intermittent abdominal pain for 3 years following previous appendectomy for the treatment of suspected appendicitis. Abdominal discomfort and pain, suggestive of intestinal obstruction, recurred after operation. A tubular mass was palpable in the right lower quadrant. Computed tomography enterography scan identified suspicious intestinal intussusception, while Tc-99m pertechnetate scintigraphy revealed a cluster of strip-like abnormal radioactivity in the right lower quadrant. On exploratory laparotomy, a tubular-shaped ileal duplication cyst was found arising from the mesenteric margin of the native ileal segment located 15 cm proximal to the ileocecal valve. Ileectomy was performed along with the removal of the duplication disease, and the end-to-end anastomosis was done to restore the gastrointestinal tract continuity. Pathological examination showed ileal duplication with ectopic gastric mucosa. The patient experienced an eventless postoperative recovery and remained asymptomatic within 2 years of postoperative follow-up.

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### INTRODUCTION

Gastrointestinal tract duplication is a rare congenital gastrointestinal malformation reported in 1733 for the first time, and termed as duplication of the alimentary tract (DAT) by Fiorani *et al*<sup>[1]</sup>. The exact etiology of DAT remains unknown, while multiple theories have been postulated, including "abortive twinning theory", "persistent embryonic diverticula theory", "split notochord theory", "intrauterine vascular accident theory" and more popular "aberrant luminal recanalization theory". More than 80% of DAT cases are diagnosed in children under 2 years but rarely in adults<sup>[2,3]</sup>. DAT can arise from any segment of the gastrointestinal tract from the mouth to the anus, but involves the ileum in most cases (44%)<sup>[4]</sup>. Clinical manifestations of DAT are highly variable, especially in adults, de-

pending on the type, size, location, and mucosal lining of the duplication. An intestinal DAT may be asymptomatic, while chief complaints consist mainly of belly pain, abdominal mass, intestinal obstruction, and hematochezia<sup>[5]</sup>. Ileal duplication occasionally becomes symptomatic until adulthood and requires subsequent medical intervention. However, ileal duplication cannot be overlooked in adult patients due to its serious complications, such as refractory bleeding, gastrointestinal perforation, and possible malignant transformation if with complicating gastric mucosa heterotopia<sup>[5]</sup>. Ileal duplication is also a diagnostic challenge in adults as it is almost impossible in the clinical setting to differentiate it from other common gastrointestinal malformations or inflammatory diseases, such as Meckel's diverticulum (MD), noncomplicating intestinal intussusception, and Crohn's disease<sup>[6]</sup>. We report a case of ileal duplication mimicking intestinal intussusception in a young man in his mid 20s, which was previously misdiagnosed as appendicitis, Crohn's disease or MD.

## CASE REPORT

A 25-year-old Han Chinese man complained of chronic abdominal pain and weight loss for 3 years. In previous hospitalization, contrast gastrointestinal radiography showed multiple mucosal filling defects in the terminal segment of the ileum, while abdominal contrast-enhanced computed tomography (CT) scan revealed localized dilation, effusion, thickening, and edema of the intestinal wall in the right lower quadrant with concomitant enlargement of multiple mesenteric lymph nodes. Ileal Crohn's disease was initially suspected but subsequently excluded due to the fact that colonoscopy only identified mild chronic inflammation. Moreover, the abdominal symptoms could not be alleviated by the medication with oral salicylazo-sulphapyridine but omeprazole. Open appendectomy was performed as indicated by a serious attack of right lower quadrant pain, and postoperative pathology showed mild simple appendicitis. However, intermittent abdominal pain free of hematochezia or melena, suggestive of intestinal obstruction, still recurred following appendectomy. The patient was referred to our department in November 2010 due to the consistently worsening abdominal symptoms in the absence of any clinically evident predisposing factors.

Physical examination revealed a 10 cm × 3 cm, tubular-shaped, soft, tender, mobile mass located in the right lower quadrant. Routine hematology and clinical chemical tests showed no clinically significant abnormalities. Repeated abdominal contrast-enhanced CT scan revealed a suspicious intestinal intussusception located in the right lower quadrant (Figure 1). Repeated contrast gastrointestinal radiography and colonoscopy identified clinically insignificant results. Tc-99m pertechnetate scintigraphy also showed a cluster of strip-like abnormal radioactivity in the right lower quadrant (Figure 2). Explorative laparotomy was indicated for suspicious MD in this patient with a history of previous abdominal surgery.

Intraoperative exploration showed no clinically sig-

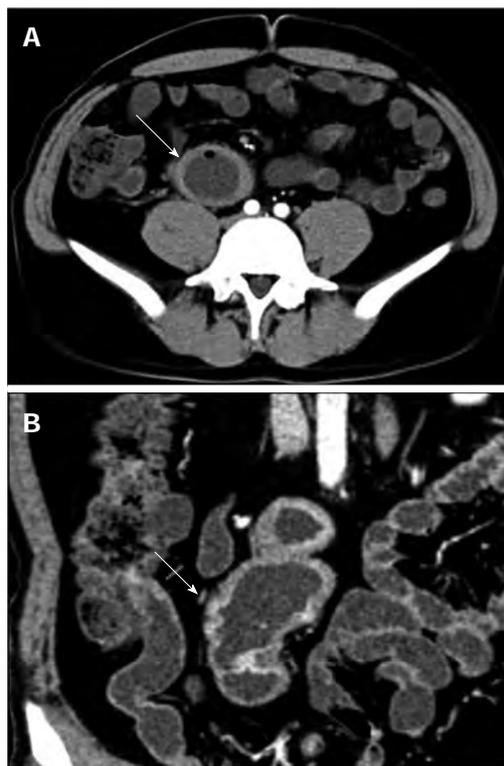


Figure 1 Computed tomography enterography scan. A: Transverse view showed suspicious ileal intussusception (white arrow) in the right lower quadrant; B: Coronal view revealed a similar result (white arrow).

nificant abnormalities, except for moderate intestinal adhesions, in the peritoneal cavity. However, a duplicating, tubular-shaped intestinal segment, 15.5 cm in length and 4 cm in diameter (Figure 3A), was found arising from the mesenteric margin of the native ileal segment located 15 cm proximal to the ileocecal valve (Figure 3B). The distal part of the ileal duplication cyst had a 2-cm, completely patent orifice into the native ileal lumen, while the proximal part ended in a blind pouch (Figure 3C). The ileal duplication cyst was easily resected along with a 7.5 cm native ileal segment, and an end-to-end ileal anastomosis was performed to restore the gastrointestinal continuity. The resection specimen showed no signs of inflammation, infection, ulceration, hemorrhage, obstruction or malignant transformation (Figure 4A). Additionally, gross pathology (Figure 4B) and histology (Figure 4C) showed that the duplication cyst was lined with ileal mucus glands and heterotopic gastric mucosae. Therefore, this disease was diagnosed as ileal duplication cyst with complicating gastric mucosa heterotopia. The patient experienced an eventless postoperative recovery, and he was discharged from hospital on postoperative day 7. The patient was followed up at the outpatient clinic and remained asymptomatic throughout a two-year follow-up period until the time of drafting this manuscript.

## DISCUSSION

DAT was firstly reported by Fitz<sup>[7]</sup> and subsequently defined by Ladd *et al*<sup>[8]</sup> as a spherical- or tubular-shaped

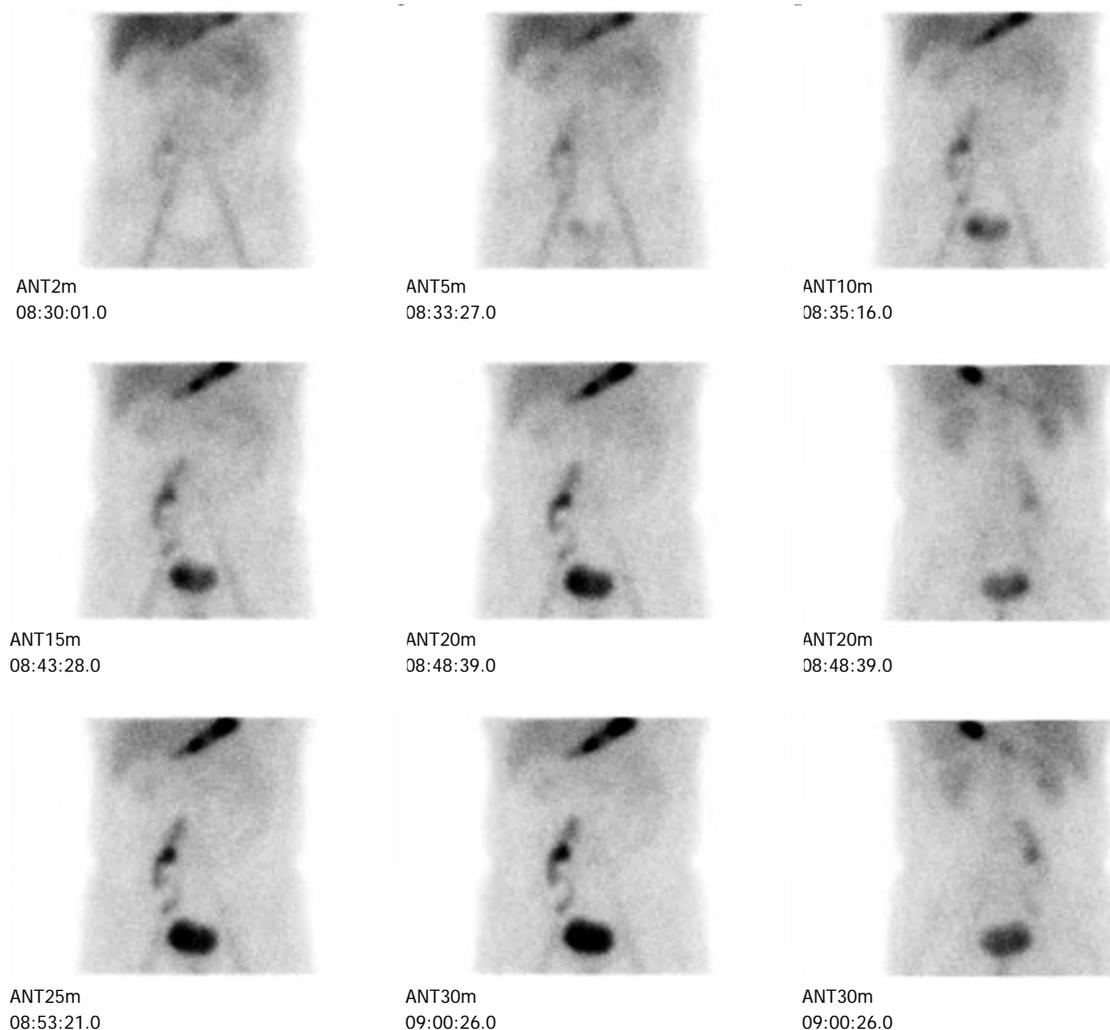


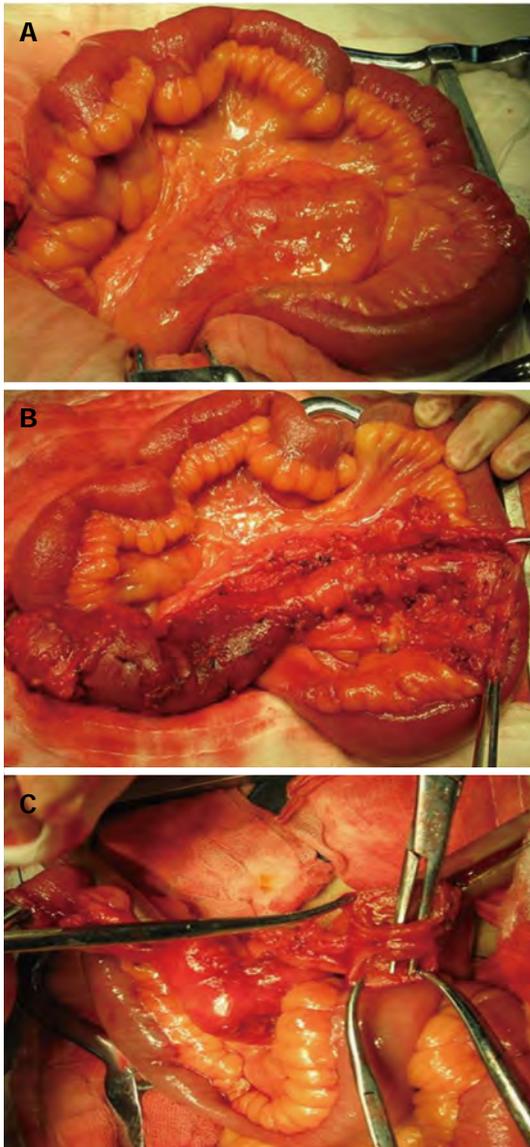
Figure 2 Tc-99m pertechnetate scintigraphy. A cluster of stripy abnormal radio-activity was located in the right lower quadrant.

anomaly that was attached or adherent to and shared the identical phenotypic characteristics with the normal alimentary tract. Although DAT is known as a rare congenital malformation (1/10000 live births), this anomaly can occur anywhere along the gastrointestinal tract, with the ileum being the most frequently affected segment<sup>[2,6]</sup>, either on the mesenteric margin or the contralateral side<sup>[9,10]</sup>. Daudet *et al*<sup>[11]</sup> reviewed 764 DAT cases, the majority of which occurred at infancy but rarely at adulthood, with a male dominance.

Ileal duplication normally exhibits highly variable and nonspecific clinical manifestations in adults. The most frequent complaints consist mainly of symptoms suggestive of gastrointestinal bleeding and intestinal obstruction<sup>[5,6,12,13]</sup>; and abdominal pain and palpable abdominal mass are also reported by approximately 50% of patients<sup>[13]</sup>. Furthermore, gastrointestinal bleeding and refractory abdominal pain may be underlain with heterotopic gastric mucosae lining the duplication cyst. Therefore, antacids can be effective in relieving abdominal pain as shown in this patient.

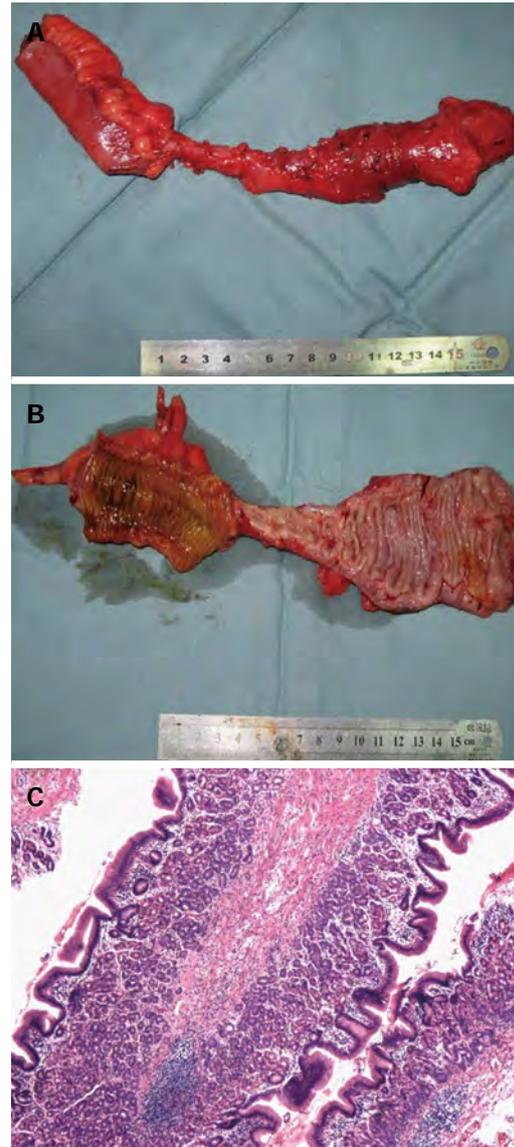
Multiple diagnostic tools are reported to be useful in the investigation of DAT, including contrast-enhanced

gastrointestinal ultrasonography and radiography, abdominal CT scan, and gastrointestinal endoscopy<sup>[14,15]</sup>. Moreover, Tc-99m pertechnetate scintigraphy is recommended as the first-line option of choice for the workup of DAT<sup>[16]</sup>. The positive result depends mainly on the abnormal enrichment of radionuclides accumulated by the heterotopic gastric mucosae. Ileal DAT mainly needs to be differentiated from MD. In pathogenesis, MD is a true congenital diverticulum deriving from the remnant of the omphalomesenteric duct during the development of the terminal ileum, while DAT can occur anywhere along the gastrointestinal tract but most frequently in the ileum; MD is normally located on the contralateral side of the mesenteric margin, while ileal duplication cyst occurs either on the mesenteric margin or the contralateral side. MD is often complicated with ectopic gastric mucosa; therefore, abdominal pain responsive to antacids is more frequently present in MD patients than in ileal DAT patients. Furthermore, MD is known to often cause a series of complications, such as diverticulitis, gastrointestinal bleeding or perforation, and intestinal obstruction, whereas these complications are relatively less common in ileal DAT. However, it is almost impossible to distinguish DAT from



**Figure 3 Exploratory laparotomy findings.** A: A 25-cm duplicating, tubular small intestinal segment was found arising from the ileal mesenteric margin; B: This duplication cyst was intimately attached to the native ileal segment located 15 cm proximal to ileocecal valve; C: this cyst had a blind end proximally and a completely patent orifice into the native ileal lumen distally.

MD prior to operation if the ileum is involved. A previous study conducted in Japan reported that only 11.2% of ileal duplication cases could be correctly diagnosed before operation, 18.2% misdiagnosed as ileal intussusception, 15.1% as ileal mass, 14.4% as ileus, and 26.7% as abdominal pain of unknown cause<sup>[17]</sup>. Therefore, it will easily lead to the misdiagnosis of ileal DAT as MD. As MD is the most frequent gastrointestinal malformation, a suspected diagnosis of MD was made and indicated for exploratory laparotomy in our case. Appendicitis also needs to be excluded as the primary complaint was right lower quadrant pain in this patient, especially if complicating infection occurred in the duplication cyst. Unfortunately, the ileal duplication, which located 15 cm proximal to the ileocecal valve, was missed in previous appendectomy. The possibility of ileal duplication should be excluded for a patient



**Figure 4 Gross and histological pathology of the resection specimen.** A: The resection specimen showed no signs of inflammation, infection, ulceration, hemorrhage, obstruction, or malignant transformation; B: The mucosal layer of the duplication cyst was lined with both small intestinal and gastric mucosae; C: Histology revealed that the duplication cyst was lined with ileal mucus glands and heterotopic gastric mucosae (hematoxylin-eosin,  $\times 100$ ).

diagnosed with suspicious MD or appendicitis but exhibiting gastrointestinal bleeding and/or intestinal obstruction. Use of laparoscopy may be helpful in identifying any suspicious ileal diseases when a diagnosis of MD or appendicitis is doubtful. Double-balloon enteroscopy may be another effective investigational technique for the diagnosis of ileal DAT if an additional ileal luminal orifice is visualized<sup>[4,18,19]</sup>.

Symptomatic treatment, such as acid-suppressing medications, may be effective in some cases if the symptoms are primarily associated with ectopic gastric mucosae. Like the possibility of adenocarcinoma in MD with complicating ectopic gastric mucosa, malignant transformation of ileal duplication cyst with complicating gastric mucosa heterotopia is also a major concern in adult patients as

epithelial instability is seen in long-standing duplication cysts. A historic review published by Johnson and his colleagues<sup>[5]</sup> reported that three out of 13 (23.1%) adult ileal DAT patients had ileal cancer, including adenocarcinoma in two patients and squamous cell carcinoma in one patient. Thus, radical resection of the duplication cyst along with the affected native intestinal segment remains the mainstay modality of definitive treatment<sup>[5,20]</sup>.

In conclusion, our report described ileal DAT, a rare congenital gastrointestinal malformation uncommonly seen in adults. This rare condition exhibits no specific manifestations although CT enterography scan and Tc-99m pertechnetate scintigraphy may identify some characteristic appearance of intussusception and MD. Surgical resection is thought to be the most effective treatment modality. Use of laparoscopy will allow a direct visualization and concomitant resection of possible ileal DAT.

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## Plastic tube-assisted gastroscopic removal of embedded esophageal metal stents: A case report

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### Abstract

A patient with stent embedding after placement of an esophageal stent for an esophagobronchial fistula was treated with an ST-E plastic tube inserted into the esophagus to the upper end of the stent using gastroscopy. The gastroscopy was guided into the esophagus through the ST-E tube, and an alligator forceps was inserted into the esophagus through the ST-E tube alongside the gastroscopy. Under gastroscopy, the stent wire was grasped with the forceps and pulled into the ST-E tube. When resistance was met during withdrawal, the gastroscopy was guided further to the esophageal section where the stent was embedded. Biopsy forceps were guided through a biopsy hole in the gastroscopy to the embedded stent to remove silicone membranes and connection threads linking the Z-shaped wire mesh. While the lower section of the Z-shaped stent was fixed by the biopsy forceps, the alligator forceps were used to pull the upper section of the metal wire until the Z-shaped metal loops elongated. The wire mesh of the stent was then removed in stages through

the ST-E tube. Care was taken to avoid bleeding and perforation. Under the assistance of an ST-E plastic tube, an embedded esophageal metal stent was successfully removed with no bleeding or perforation. The patient experienced an uneventful recovery after surgery. Plastic tube-assisted gastroscopic removal of embedded metal stents can be minimally invasive, safe, and effective.

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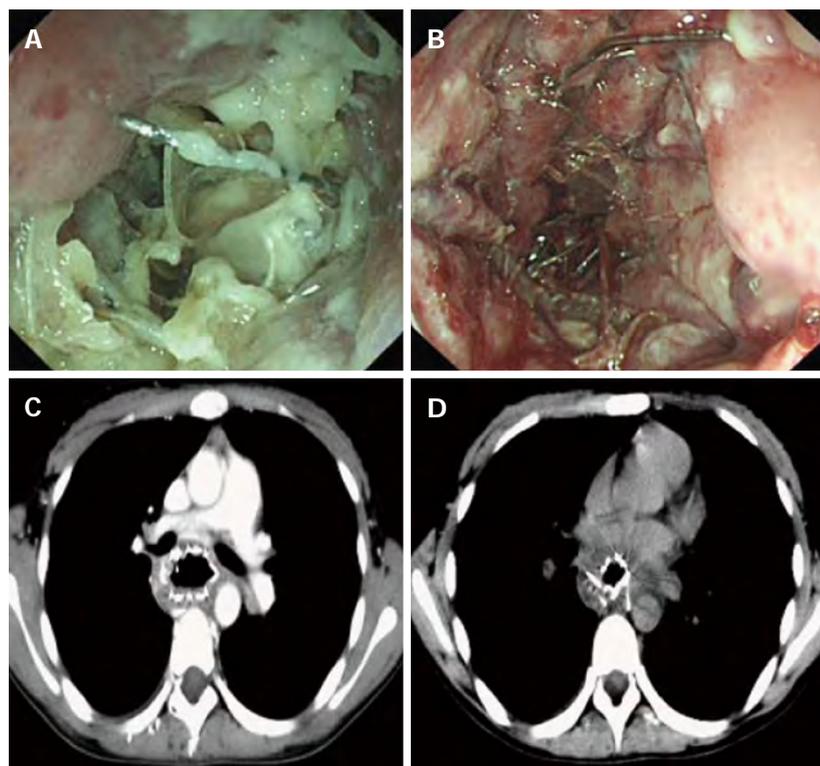
**Key words:** Esophagus; Stents; Gastroscopy; Complication

**Core tip:** A patient presented with a disordered stent structure as a result of failure of repeated attempts at gastroscopic removal. An ST-E tube was inserted into the esophagus. An alligator forceps was inserted into the ST-E tube alongside a gastroscopy. The stent wire was gripped and pulled up into the tube. Biopsy forceps were inserted into the lower section of the stent through the biopsy hole to fix the stent, while the alligator forceps continued to be used to pull up the stent wire until the Z-shaped metal loops became elongated stripes. All the stent wire was removed through the ST-E tube.

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### INTRODUCTION

The use of fully covered self-expandable esophageal metal stents has favorable results in treating a variety of



**Figure 1** Disordered stent structure as a result of failure of repeated attempts at gastroscopic removal of the stent. A-D: Enhanced chest computed tomography scan performed showed the stent was situated in the middle section of the esophagus after esophageal stent placement, the wall of the middle and inferior segments of the esophagus thickened noticeably, part of the stent was embedded into the esophageal wall, borders between the stent and surrounding fat were blurred, and the upper end of the stent pressed against the trachea carina.

benign and malignant esophageal strictures and esophageal fistulae<sup>[1-6]</sup>. However, stent placement over a prolonged period can result in hyperplastic tissue overgrowth on both ends of the stent, leading to in-stent restenosis<sup>[7-9]</sup>. Therefore, stents should be removed after an appropriate period after treatment of benign esophageal strictures and fistulae<sup>[10-12]</sup>. Notable tissue hyperplasia can occur at both stent ends, causing difficulty in stent removal and sometimes requiring surgical treatment. This procedure has a high risk of trauma<sup>[13,14]</sup>. Therefore, a minimally invasive, low-risk method is needed for removal of embedded esophageal stents. Here, we report our experience with a novel approach to gastroscopic removal of an embedded esophageal stent.

## CASE REPORT

The patient was a 15-year-old girl who had experienced coughing after drinking since early March 2012. The imaging of iodinated contrast-enhanced radiological examination showed an esophagobronchial fistula arising from the left bronchus to the middle portion of the esophagus. Under gastroscopy on May 30, 2012, a fully covered Z-shaped metal stent measuring 2 cm × 6 cm was placed within the esophagus to cover the fistula opening. After the procedure, the coughing after drinking disappeared. However, the patient developed esophageal obstruction. Gastroscopy on August 13, 2012 showed tissue hyperplasia on both stent ends, luminal stenosis, and embedding of both stent ends in the hyperplastic tissue.

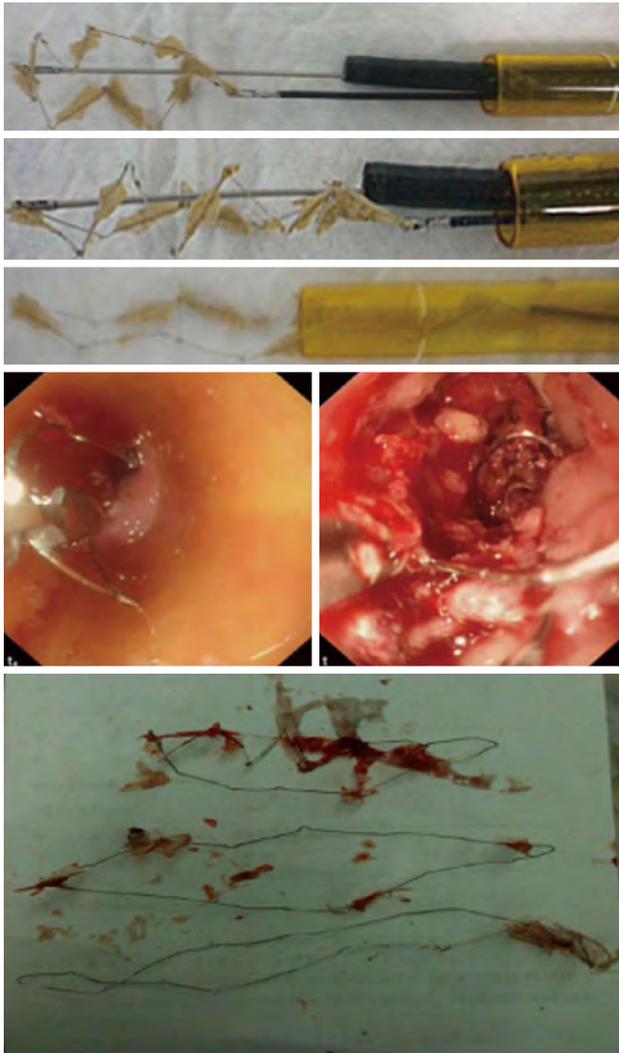
Attempts to remove the stent under endoscopy failed and led to a disorganized stent structure. Enhanced chest computed tomography scan showed the wall of

the middle and inferior segment of the esophagus were noticeably thickened. Part of the stent was embedded in the esophageal wall, the boundary between the stent and surrounding fat was blurred, and the upper end of the stent was pressed against the trachea carina (Figure 1). After consultation with thoracic surgeons, we decided to perform gastroscopic removal on August 31, 2012.

Under the guidance of gastroscopy, an ST-E plastic tube (3 cm × 40 cm) was inserted into the esophagus to the upper end of the stent. A gastroscope was guided into the esophagus through the ST-E tube, and an alligator forceps was inserted into the esophagus through the ST-E tube alongside the gastroscope. Under gastroscopy, the stent wire was gripped with the forceps and pulled into the ST-E tube. When resistance was met during withdrawal, the gastroscope was guided further to the esophageal section where the stent was embedded. Biopsy forceps were sent through a biopsy hole in the gastroscope and inserted near the embedded stent to remove the silicone membranes and connection threads linking the Z-shaped stent wire. Next, while the lower section of the Z-shaped stent was fixed by the biopsy forceps, the alligator forceps were used to pull the upper section of the metal wire until the Z-shaped metal loops elongated. The wire mesh of the stent was then removed in stages through the ST-E tube (Figure 2).

## DISCUSSION

Benign esophagobronchial fistulae are rare and often result from trauma, esophageal spontaneous rupture, tuberculosis, and Crohn's disease<sup>[2,4,5]</sup>. Treatment is usually difficult and surgical interventions involve a high risk of



**Figure 2** An alligator forceps was inserted into an ST-E tube alongside a gastroscop and, under the guidance of gastroscopy, the stent wire was gripped and pulled up into the ST-E tube. Biopsy forceps were inserted into the lower section of the Z-shaped stent through a biopsy hole in the gastroscop to fix the stent; in the meantime, the alligator forceps continued to be used to pull up the stent wire until the Z-shaped metal loops became elongated strips. All the stent wire was removed in stages through the ST-E tube.

trauma. Placement of fully covered self-expanding metal stents has become a viable treatment option. However, stent placement over a prolonged period can result in hyperplastic tissue overgrowth on both ends of the stent, leading to in-stent re-stenosis. Removing embedded stents is difficult. One study attempted to remove a stent by placing a secondary stent within the primary stent<sup>[13]</sup>. However, in this case, the stent structure was compromised and became disordered during attempts to remove the stent under endoscopy, with the result that sharp parts of the stent were entering the esophageal wall. Thus, placement of another stent might have caused esophageal perforation and damage to surrounding organs.

In our view, two goals must be achieved to successfully remove a structurally disordered Z-shaped metal stent embedded in the esophageal wall under

gastroscopy. First, the esophageal entrance and throat must be protected from scratching by the stent wire during removal; second, each of the Z-shaped stent loops needs to be pulled outward until it is elongated. Gastroscopic procedures conducted through a ST-E tube meet these requirements, as the plastic tube fully protects the upper portion of the esophagus and throat from scratching by the stent wire. A two-handed operation is possible with an ST-E tube: with one hand, an alligator forceps is inserted into the esophagus through the ST-E tube alongside the gastroscop. Under the guidance of gastroscopy, the stent wire is gripped and pulled outward into the ST-E tube. If resistance is met during pulling, the gastroscop can be sent further to the esophageal section where the stent is embedded and biopsy forceps guided with the second hand through a biopsy hole in the gastroscop to insert into the embedded stent to remove silicone membranes and connection threads linking the Z-shaped stent. While the lower section of the Z-shaped stent is fixed by the biopsy forceps, the alligator forceps can be used to pull the upper section of the metal wire until the Z-shaped metal loops are elongated, to enable the wire mesh of the stent to be removed through the ST-E tube. The wire mesh of the stent is removed in stages by repeating the above procedures.

In our view, plastic tube-assisted gastroscopy is a minimally invasive, safe, and effective method for removal of esophageal embedded metal stents.

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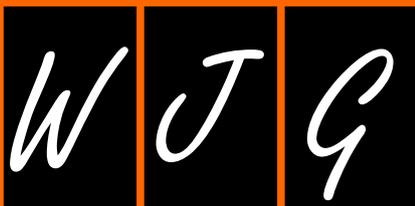
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## Strategies to reduce pulmonary complications after esophagectomy

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### Abstract

Esophagectomy, the surgical removal of all or part of the esophagus, is a surgical procedure that is associated with high morbidity and mortality. Pulmonary complications are an especially important postoperative problem. Therefore, many perioperative strategies to prevent pulmonary complications after esophagectomy have been investigated and introduced in daily clinical practice. Here, we review these strategies, including improvement of patient performance and technical advances such as minimally invasive surgery that have been implemented in recent years. Furthermore, interventions such as methylprednisolone, neutrophil elastase inhibitor and epidural analgesia, which have been shown to reduce pulmonary complications, are discussed. Benefits of the commonly applied routine nasogastric decompression, delay of oral intake and prophylactic mechanical ventilation are unclear, and many of these strategies are also evaluated here. Finally, we will

discuss recent insights and new developments aimed to improve pulmonary outcomes after esophagectomy.

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**Key words:** Esophagectomy; Complications; Pneumonia; Acute lung injury; Acute respiratory distress syndrome

**Core tip:** Pulmonary complications following esophagectomy significantly contribute to postoperative morbidity and mortality. Over the years many strategies aimed at reducing pulmonary complications have been investigated. In the current article, we discuss these strategies, specifically minimally invasive surgical techniques; anti-inflammatory therapies and optimization of patient performance.

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### INTRODUCTION

Esophageal cancer is the sixth leading cause of cancer related mortality, and its incidence is increasing rapidly<sup>[1]</sup>. For patients with loco-regional disease the best chance for long-term survival is offered by a transthoracic esophagectomy after neoadjuvant therapy<sup>[2-4]</sup>. However, esophagectomy is considered to be one of the most invasive and complex gastrointestinal procedures with a high post-operative morbidity and mortality<sup>[5]</sup>. Concentration of surgical treatment in high volume centers and improvements in perioperative care have led to significant reductions in postoperative mortality and improved long-

term survival<sup>[5,6]</sup>.

Respiratory complications are most common after esophagectomy, with up to a 60% incidence rate; respiratory failure due to pulmonary complications remains the major cause of postoperative morbidity and mortality after esophagectomy<sup>[7,8]</sup>. A wide range of perioperative strategies have been introduced in order to reduce these pulmonary complications. In this editorial we will discuss several of these strategies.

## DEFINITIONS

The most severe pulmonary complications following esophagectomy are pneumonia, adult respiratory distress syndrome (ARDS) and acute lung injury (ALI). Pneumonia is the most common complication and is significantly associated with need for re-intubation, prolonged hospital stays and in hospital mortality<sup>[9]</sup>. Although ARDS and ALI have been clearly defined during American-European consensus conferences, criteria for pneumonia differ widely<sup>[10-12]</sup>. In a recent systematic review, pneumonia rates were reported by 56 studies and defined by 18 studies. However, 16 different definitions were used, resulting in a wide range of reported pneumonia rates (between 1.5% and 38.9%). Consequently, this variation makes it difficult to compare study results<sup>[10]</sup>. Therefore, generating a consensus on the definition of pneumonia after esophagectomy is an important step in improving the quality and comparability of research. Despite the heterogeneity in definitions, several interesting strategies to reduce pulmonary complications after esophagectomy have been described.

## OPTIMISATION OF PERFORMANCE STATUS

### Nutrition

Improvement of performance status of patients undergoing esophagectomy is important in reducing pulmonary complications. Adequate enteral nutrition is an important tool to achieve this in the pre-operative and postoperative phase. When nutrition is inadequate, leading to malnutrition, this is associated with expiratory muscle weakness and pulmonary complications after major upper abdominal surgery<sup>[13]</sup>. Preoperative malnutrition also increases the risk for overall complications after esophagectomy (OR = 3.50, 95%CI: 1.89-6.49)<sup>[14]</sup>. Furthermore, when all patients undergoing esophagectomy receive preoperative intensive nutritional support by a dietician, fewer postoperative complications are observed (OR = 0.23, 95%CI: 0.05-0.97)<sup>[15]</sup>. This is supported by another prospective cohort study that investigated preoperative nutritional support for malnourished patients<sup>[16]</sup>. Despite the fact that preoperative nutritional support seems a logical and promising strategy to prevent postoperative pulmonary complications, clear evidence is lacking.

An important role for nutrition also exists in the postoperative phase. Early enteral nutrition after gastro-

intestinal surgery improves patient recovery and reduces morbidity and mortality<sup>[17]</sup>. However, commonly a nil-by-mouth regimen is still applied after esophagectomy. The rationale for this regimen is the concern that early oral intake would result in vomiting with subsequent aspiration pneumonia. Furthermore sequelae of anastomotic leakage are thought to be more severe if leaked fluids contain food besides to saliva. However, benefits of a nil-by-mouth regimen are theoretical and evidence is lacking<sup>[18]</sup>.

Jejunal tube feeding can be started early to ensure enteral nutrition following esophagectomy. Compared to total parenteral nutrition or fasting this reduces postoperative pneumonia rates by 50% or more<sup>[19,20]</sup>. Drawbacks are frequent dislocation of nasojejunal tubes, and serious complications such as leakage<sup>[21]</sup>.

The risks of artificial feeding, combined with the lack of evidence concerning effects of a nil-by-mouth regimen, are reasons to investigate the feasibility and safety of starting oral intake early after esophagectomy. Interestingly, for major upper abdominal surgery early oral intake has already been demonstrated to be feasible and safe<sup>[22]</sup>. However, further research is needed to provide more evidence in patients undergoing esophagectomy.

### Inspiratory muscle training

Another method to optimize performance status is through physical exercise. If postoperatively compromised, respiratory muscle strength will result in reduced lung function and insufficient coughing. This might induce atelectasis, which, acting in combination with postoperative pain and sedation, might result in hypoxia<sup>[23]</sup>. For this reason, several studies have been performed to prevent postoperative decrease in muscle function by preoperative physiotherapy. For example, a large-scale randomized controlled trial (RCT) demonstrated that inspiratory muscle training (IMT), for two or more weeks before coronary artery bypass graft surgery reduced the incidence of all pulmonary complications from 35% to 18%, and for pneumonia from 16% to 7%<sup>[23]</sup>. Preoperative IMT is also feasible for patients undergoing esophagectomy, and even preserves postoperative respiratory muscle strength<sup>[24,25]</sup>.

### Minimizing irradiated lung volume

Patients with esophageal cancer are mostly treated neoadjuvant with radiotherapy and chemotherapy<sup>[4]</sup>. However, these multimodality treatments are often correlated with an increase in postoperative pulmonary complications and mortality<sup>[26]</sup>. An adjustable factor in these treatments is the amount of radiation on the lung. For example, when  $\geq 40\%$  of the lung volume received  $\geq 10$  Gy, the incidence of pneumonia and ARDS significantly increased from 8% to 35%<sup>[26]</sup>. Multivariate analysis of various dosimetric factors has shown that the total amount of lung spared from doses  $> 5$  Gy is significantly correlated with reduced pulmonary complications<sup>[27]</sup>. Though this correlation is not found in all studies, it seems reasonable to reduce the amount of irradiated healthy lung

**Table 1 Advantages of prone positioning**

Alveolar recruitment
Improved redistribution of ventilation
Redirection of compressive force of the heart
Better clearance of secretion
Lung retraction not necessary
Shorter operation time
Fewer ports needed

tissue from an oncological viewpoint.

## PEROPERATIVE STRATEGIES

### *Minimally invasive surgery*

Minimally invasive surgery has rapidly evolved in recent years. Since minimally invasive approaches reduce factors associated with pulmonary complications (*e.g.*, blood loss, pain, and inflammation), minimally invasive esophagectomy would be especially beneficial with respect to pulmonary complications<sup>[28]</sup>. Recently, a prospective RCT demonstrated the benefits of a minimally invasive approach regarding pulmonary complications for the first time<sup>[29]</sup>. Fifty-nine patients undergoing thoracoscopic esophagectomy in prone positioning were compared to 56 patients undergoing open transthoracic esophagectomy in a left semi-lateral position. The pneumonia (clinical diagnosis confirmed by radiologic investigation and a positive sputum culture) rate within the first two postoperative weeks was 9% *vs* 29% in the open group (RR = 0.30, 95%CI: 0.12-0.60). Since a sputum culture is often negative in case of pneumonia, this study may underestimate the true pneumonia rate. However, the observed reduction in postoperative pneumonia by the minimal invasive approach is significant<sup>[29]</sup>.

It is questionable whether the minimally invasive approach, the prone positioning, or a combination of both caused the outcomes in this trial. Traditionally, patients undergo an open transthoracic esophagectomy in a left lateral decubitus position with double lumen tube intubation for one-lung ventilation. However, with the development of minimally invasive, thoracoscopic techniques, patient positioning was no longer restricted to a lateral decubitus position giving rise to minimally invasive, thoracoscopic, prone position techniques<sup>[30]</sup>. There are several advantages to a prone positioning, including partial or intermittent single lumen ventilation, as opposed to total lung collapse by a double lumen intubation in lateral decubitus position (Table 1). Further, perioperative distribution of pulmonary ventilation and circulation might be improved, leading to better oxygenation<sup>[30]</sup>. These advantages translate in improved postoperative outcomes, as shown by two studies that demonstrated an advantage of prone positioning compared to left lateral decubitus positioning<sup>[31,32]</sup>.

Despite these advantages, prone positioning has not been adopted widely. Surgeons question whether or not safety is compromised due to the difficulty of an emer-

gency conversion in prone position to left lateral with subsequent difficult airway management. However, a recent systematic review concluded prone positioning to be safe<sup>[30]</sup>. Furthermore in the previously mentioned trial during thoracoscopic dissection all patients were in prone position<sup>[29]</sup>.

### *Corticosteroids and neutrophil elastase inhibitors*

Pulmonary complications can be reduced by dampening the inflammatory response through medication. Sato *et al.*<sup>[33]</sup> found a pre-operative single dose of methylprednisolone (10 mg/kg) significantly reduced postoperative inflammation and subsequent pulmonary complications (from 30% to 9%). Other studies found similar benefits of methylprednisolone, without observing adverse effects<sup>[34]</sup>.

However, even with pre-operative methylprednisolone administration, pulmonary complications occur frequently<sup>[35]</sup>. This might be caused by the systemic inflammatory response on esophagectomy, leading to accumulation of neutrophils in the lungs. Subsequently local release of neutrophil elastase injures the lung<sup>[36]</sup>. Since glucocorticoids do not affect the release or function of neutrophil elastase, additional selective inhibition of neutrophil elastase might be beneficial<sup>[37]</sup>. Indeed, adding a selective neutrophil elastase inhibitor to methylprednisolone improves oxygenation during the first seven postoperative days<sup>[38]</sup>. Furthermore, perioperative selective neutrophil elastase inhibition prevented ALI after minimally invasive esophagectomy<sup>[36]</sup>.

The results of perioperatively administered methylprednisolone and neutrophil elastase inhibitors are encouraging. However, all trials were conducted in Eastern populations. Because genomic factors might influence results, trials should be conducted in other populations in order to determine whether these results can be extrapolated to all populations.

### *Protective ventilation*

Protective ventilation can reduce the amount of mechanically induced pulmonary injury during esophagectomy. During protective ventilation, tidal volumes are reduced and a moderate positive end-expiratory pressure is applied<sup>[39]</sup>. This strategy reduces inflammation and improves oxygenation compared to conventional ventilation. Though pneumonia rates have shown to be lower after protective ventilation, this was not significantly different<sup>[39]</sup>.

### *Goal-directed fluid therapy*

Goal directed fluid administration reduces postoperative pulmonary complications in other types of surgery such as major (upper) abdominal and major vascular surgery (RR = 0.7, 95%CI: 0.6-0.9)<sup>[40]</sup>. With this strategy, fluids are administered to achieve predefined, patient-specific hemodynamic goals, avoiding excessive resuscitation or under-resuscitation as seen with liberal or restrictive fluid administration<sup>[40]</sup>. Increased volume of perioperative fluid administration increases the risk for pulmonary complica-

tions following esophagectomy<sup>[41]</sup>. Therefore, it would be interesting to determine if this can be prevented by goal-directed fluid administration. However, because instruments that adequately measure hemodynamic parameters to guide fluid administration are invasive or difficult to use, they are not commonly applied. As a consequence several simple, minimally invasive instruments have been developed. Further research should first compare these devices in order to determine which impacts outcomes most<sup>[42]</sup>.

## POSTOPERATIVE STRATEGIES

Strategies to reduce pulmonary complications that are applied postoperatively are analgesia, prolonged postoperative ventilation, and nasogastric decompression. Adequate postoperative analgesia is important after esophagectomy, because postoperative pain from thoracic and upper abdominal wounds compromises pulmonary function, coughing, and mobilization, resulting in atelectasis and pneumonia. In patients undergoing esophagectomy, thoracic epidural analgesia is more effective than intravenous opioid analgesia<sup>[43]</sup>. Furthermore, thoracic epidural analgesia facilitates early extubation and reduces the risk for respiratory failure, overall pulmonary complications and mortality<sup>[9,44]</sup>.

Postoperative pain, aspiration and airway edema were the main rationale for routinely performing prolonged postoperative ventilation for many years. Mechanical ventilation could cause barotrauma, ventilator acquired pneumonia and endotracheal tube related problems. Early extubation, based on individual clinical factors, does not increase pulmonary complications<sup>[45]</sup>. After early extubation, routine bronchoscopic clearance of secretions was associated with reduced mortality, possibly due to preventing of postoperative pulmonary complications<sup>[44]</sup>. However, further studies are needed to substantiate this retrospectively found effect.

Another commonly applied strategy to reduce pulmonary complications due to postoperative aspiration is routine nasogastric decompression. However, a recent meta-analysis showed that after major upper abdominal surgery this strategy increased pulmonary complications (OR = 1.49, 95%CI: 1.01-2.21)<sup>[46]</sup>. Routine insertion of a nasogastric tube six to ten days following a esophagectomy is not beneficial compared to early removal of the nasogastric tube (second day postoperative)<sup>[47]</sup>. Furthermore, the commonly used single lumen nasogastric tube does not reduce aspiration compared to the situation in which no tube is routinely inserted<sup>[48,49]</sup>. In addition, routine nasogastric decompression failed to reduce pneumonia rates<sup>[48,49]</sup>. However, trials that have investigated routine nasogastric tube insertion did not specifically investigate pulmonary complications, highlighting a need for a trial to detect a clinically significant reduction in pulmonary complications is needed.

## CONCLUSION

Pulmonary complications are an important problem after esophagectomy. However, many advances have been made in recent years. Proven effective strategies are minimally invasive surgery, thoracic epidural analgesia and early enteral nutrition. Perioperative methylprednisolone and neutrophil elastase inhibitor administration can be added to these strategies if their benefits are confirmed in additional studies.

Preoperative optimization of performance status, prone positioning and targeted fluid therapy are promising for further research. While new interventions are extensively investigated before application, it seems unjust to apply invasive interventions without proven benefits. Therefore several commonly applied strategies (*e.g.*, routine nasogastric decompression, delay of oral intake, prophylactic mechanical ventilation) are currently being re-evaluated.

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## Risk prediction of hepatitis B virus-related hepatocellular carcinoma in the era of antiviral therapy

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## Abstract

Chronic hepatitis B (CHB)-related hepatocellular carcinoma (HCC) is a major health problem in Asian-Pacific regions. Antiviral therapy reduces, but does not eliminate the risk of HCC. It would be a heavy financial burden in most low and middle economic countries if all CHB patients received antiviral therapy and HCC surveillance. Thus, there is a need for accurate risk prediction to assist prognostication, decisions on the need for antiviral therapy and HCC surveillance. A few well-established risk factors for HCC, namely advanced age, male gender, high viral load, cirrhosis *etc.*, are the core components of three HCC risk scores: CU-HCC, GAG-HCC and REACH-B scores. These 3 scores were confirmed to be accurate in predicting HCC up to 10 years in treatment-naïve patients. Their validity and applicability have recently been demonstrated in a large cohort of entecavir treatment patients. A decrease in risk scores after antiviral therapy translates to a lower risk of HCC. These findings support the application of HCC risk scores in all CHB patients. Different levels of care and different intensities of HCC surveillance should be offered according to the risk profile of patients. Patients at risk of HCC should undergo regular HCC surveillance,

even when they are receiving antiviral treatment.

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**Key words:** Antiviral therapy; Cirrhosis; Hepatitis B virus DNA; Hepatocellular carcinoma; Risk prediction score; Transient elastography

**Core tip:** CU-hepatocellular carcinoma (HCC), GAG-HCC and REACH-B scores accurately predict subsequent HCC development in both treatment-naïve patients with chronic hepatitis B and those receiving antiviral therapy. At the recommended cutoff values, baseline CU-HCC and REACH-B scores had high sensitivity, while the GAG-HCC score had high specificity in predicting HCC. Patients persistently in the low-risk category have the lowest risk of HCC; those "downgraded" in risk category have significantly lower, but a small risk of HCC compared to those in the high-risk category. Patients in the high-risk category either at baseline or after treatment should undergo HCC surveillance.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer death in men worldwide<sup>[1]</sup>. Chronic hepatitis B virus (HBV) infection is one of the major causes of HCC, and it is estimated that over 350 million people are chronically infected with HBV worldwide<sup>[2]</sup>. Globally, HBV accounts for 53% of all cases of HCC<sup>[3]</sup>. Due to the high preva-

lence of HBV infection, the incidence of HCC in Eastern Asia and Southeast Asia is the highest in the world<sup>[4]</sup>.

In the last two decades, the development of antiviral therapy was a major breakthrough in the management of chronic hepatitis B (CHB), which modifies the natural history of the disease and reduces the risk of HCC<sup>[5-7]</sup>. Nonetheless, there is still a low, but clinically relevant risk of HCC in patients receiving antiviral therapy. It would be a heavy financial burden, particularly in low and middle economic countries, if all CHB patients received antiviral therapy and HCC surveillance. Thus, there is a need for accurate risk prediction to assist prognostication, decisions on the need for antiviral therapy and HCC surveillance.

## RISK FACTORS FOR HBV-RELATED HCC

### Treatment-naïve patients

A handful of factors have been repeatedly shown to increase the risk of HCC when studying the natural history of chronic HBV infection. In general the risk factors can be categorized into host factors, liver factors and viral factors (Table 1). Host factors include advanced age<sup>[8-10]</sup>, male gender<sup>[9,10]</sup>, family history of HCC<sup>[11]</sup>, and possibly single-nucleotide polymorphisms at different human genomic loci [*e.g.*, chromosome 1p36.22, chromosome 6 of human leukocyte antigen (HLA)-DP and HLA-DQ loci, and chromosome 8p12]<sup>[12,13]</sup>. Immunosuppressed conditions like human immunodeficiency virus co-infection is another risk factor<sup>[14]</sup>. Liver factors consist of advanced fibrosis and cirrhosis<sup>[11]</sup>; poor liver function as evidenced by hypoalbuminemia and hyperbilirubinemia<sup>[8]</sup>; active hepatitis as evidenced by high alanine aminotransferase (ALT) and active necroinflammation demonstrated on liver biopsy<sup>[9]</sup>; and other concomitant liver diseases such as co-infection with hepatitis C virus or hepatitis delta virus, alcoholic liver disease and nonalcoholic fatty liver disease<sup>[11]</sup>. Viral factors include high serum HBV DNA level<sup>[8,15]</sup>, hepatitis B virus e antigen (HBeAg) seropositivity<sup>[16]</sup>, HBV genotype C<sup>[17]</sup> and subgenotype Ce<sup>[18]</sup>, core promoter mutations<sup>[10]</sup> and probably high serum hepatitis B surface antigen (HBsAg) level<sup>[19]</sup>.

### Patients receiving antiviral therapy

The natural history of chronic HBV infection is altered by antiviral therapy. Therefore, the risk factors for HCC may be different in treated patients compared to untreated patients. The landmark Asian lamivudine trial did not specifically determine the risk factors for HCC, however, baseline Child-Pugh and Ishak fibrosis score, as well as genotypic resistance *YMDD* mutation were risk factors for disease progression<sup>[6]</sup>. The drug-resistant mutant did not increase the risk of HCC (both 4% in patients with or without *YMDD* mutation). Nonetheless, the significance of *YMDD* mutation might be masked by the short follow-up duration (study prematurely terminated at 32 mo) and the unspecified interval between emergence of drug resistance and HCC development.

**Table 1 Risk factors for hepatitis B virus-related hepatocellular carcinoma**

Host factors	Liver factors	Viral factors
Advanced age	Advanced fibrosis	High serum HBV DNA
Male gender	Cirrhosis	Positive HBeAg
Family history of HCC	Hypoalbuminemia	HBV genotype C
SNP at human genomic loci, <i>e.g.</i> Chromosome 1p36.22	Hyperbilirubinemia	HBV subgenotype Ce
Chromosome 6 of HLA-DP/Q loci	High ALT	Core promoter mutations
Chromosome 8p12	Active necroinflammation	High serum HBsAg level
Immunosuppressed condition, <i>e.g.</i>	Concomitant liver diseases, <i>e.g.</i>	
Human immunodeficiency virus co-infection	Hepatitis C virus co-infection	
	Hepatitis delta virus co-infection	
	Alcoholic liver disease	
	Nonalcoholic fatty liver disease	

ALT: Alanine aminotransferase; HBeAg: Hepatitis B virus e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen; SNP: Single-nucleotide polymorphism.

In a retrospective study of 2795 Japanese CHB patients (657 lamivudine-treated *vs* 2138 untreated patients), the absence of treatment, male gender, family history of HBV carriage, age greater than 40 years, fibrosis more than grade 2 of 4, albumin level below 40 g/L, and platelet count of < 150000/mm<sup>3</sup> were independent risk factors for HCC<sup>[20]</sup>. The risk factors identified in this study appeared to be no different from those identified from studies on the natural history, probably because more than 75% of patients were untreated.

In a nationwide study from Greece retrospectively analyzing 818 HBeAg-negative patients treated with lamivudine, advanced age and cirrhosis were risk factors for HCC<sup>[21]</sup>. On-therapy virologic remission (*i.e.*, undetectable on-treatment serum HBV DNA level) did not significantly affect the incidence of HCC (although there was a trend for lower risk of HCC in the absence of cirrhosis). As all patients with on-therapy virologic remission who developed HCC (8 of 228; 3.6%) occurred within 30 mo of lamivudine treatment, some of these tumors might have been pre-existing HCC.

A recent large-scale cohort study of 1531 entecavir treatment CHB patients demonstrated the importance of maintained virologic response<sup>[22]</sup>. Old age, cirrhosis, and virologic remission for 24 mo or more were independent factors associated with HCC in the entire cohort; whereas advanced age and hypoalbuminemia were predictors in non-cirrhotic patients. Although maintained virologic response was important, 30 out of 47 patients (64%) who achieved this virologic target still developed HCC. This can be explained by the early integration of HBV into the host genome and the presence of cirrhosis, such that

even with very effective suppression of viral replication with antiviral agents, HCC may still develop<sup>[23]</sup>.

Summarizing the findings of these studies, advanced age and cirrhosis are the two major risk factors consistently demonstrated in patients receiving antiviral therapy. While maintained virologic response is likely a protective factor, baseline HBV DNA level is no longer important in these treated patients as it is usually much reduced after treatment. Theoretically HBsAg level, which reflects the amount and transcriptional activity of covalently closed circular DNA inside the liver, might have a role in predicting HCC in treated patients when serum HBV DNA is no longer detectable<sup>[24]</sup>. However, this was not confirmed in patients receiving entecavir<sup>[22]</sup>. The probable reason for this is that these patients had active disease to start with; those with lower HBsAg levels were more likely to be cirrhotic. In other words, there were no “inactive HBV carriers” at very low risk of HCC as in untreated natural history cohorts<sup>[19]</sup>.

## APPROACHES TO DEVELOP RISK SCORES

There are different approaches to developing a risk score for HCC, however, the first common step is to identify important independent factors associated with HCC in a training cohort. After statistical analysis, scores are assigned to different parameters in the equation to make up the final score. In order to demonstrate the applicability and reproducibility of the score, it should be validated in an independent cohort. If this independent cohort is not available, the leave-one-out cross-validation can be applied to assess the performance of the score in new data<sup>[25]</sup>. This validation involves using a single observation from the original sample as the validation data, and the remaining observations as the training data. This is repeated such that each observation in the sample is used once as the validation data.

Take the CU-HCC score as an example, significant variables were first identified in the multivariable Cox proportional hazards model<sup>[8]</sup>. A score was attributed to each variable according to its relative contribution in the model, as determined by the  $\chi^2$  score. Furthermore, different cutoff values of the score were determined to categorize patients into different levels of risk (*i.e.*, low-, medium-, and high-risk categories). The performance of the cutoff can be assessed in terms of discriminatory ability and monotonicity by the linear trend  $\chi^2$  test<sup>[26]</sup>.

Validation of the score usually involves two steps: discrimination and calibration. Discrimination can be assessed with the receiver operating characteristic (ROC) curve, *i.e.*, area under ROC (AUROC) curves, sensitivity, and specificity. Calibration is evaluated by estimating the observed HCC risk using the Kaplan-Meier method with the same cumulative risk scores. A combination of neighboring groups of cumulative risk scores will be performed if the observed HCC risk in a group with the same cumulative risk score is low<sup>[9]</sup>.

## EXISTING PREDICTION SCORES FOR HCC

The three most commonly applied HCC risk scores are described below (Tables 2 and 3).

### CU-HCC score

The CU-HCC score<sup>[8]</sup> was first derived from a cohort of 1005 Chinese CHB patients from a prospective study on the surveillance of HCC in chronic HBV carriers from The Chinese University of Hong Kong (abbreviated as CU in the name of the score)<sup>[18]</sup>. It was validated in an independent cohort of 424 Chinese CHB patients<sup>[27]</sup>. Both cohorts were from tertiary referral clinics. While all patients were treatment-naïve at baseline, 15.1% and 25.0% of patients from the training and validation cohort, respectively, received antiviral therapy during the long-term follow up to 10 years. The CU-HCC score is composed of 5 parameters: age, albumin, bilirubin, HBV DNA, and cirrhosis; it ranges from 0 to 44.5 (Table 2). The investigators identified two cutoff values (5 and 20) which best discriminated HCC risk into three categories. The 5-year HCC-free survival rates were 98.3%, 90.5%, and 78.9% in the low-, medium-, and high-risk groups, respectively. By applying the lower cutoff value, this score has high negative predictive value of 97.8% to exclude future HCC development.

### GAG-HCC score

The GAG-HCC score<sup>[10]</sup> was first developed from a cohort of 820 Chinese CHB patients from tertiary referral clinics. The name was abbreviated from “Guide with Age, Gender, HBV DNA, Core promoter mutations and Cirrhosis”. All patients were treatment-naïve at baseline and censored at the time of initiation of antiviral therapy. As an independent cohort was not included, the investigators adopted the leave-one-out cross-validation mentioned above<sup>[25]</sup>. There are two versions of the score. The original version is composed of gender, age, core promoter mutations, HBV DNA level and cirrhosis. There is a simplified version which omits core promoter mutations, as they may not be easily available in some centers. The score ranges widely to above 100, as age (in years) is one of the components in the formula. A cutoff value of 101 was found to have good sensitivity and specificity of 84.1% and 76.2% for 5-year prediction, and 88.0% and 78.7% for 10-year prediction, respectively. The negative predictive values were as high as 98.3% to 100% to exclude future HCC development.

### REACH-B score

The REACH-B score<sup>[9]</sup> was first derived from a cohort of 3584 Chinese CHB patients from the community-based prospective Taiwanese REVEAL-HBV study<sup>[15]</sup>, and then validated in a cohort of 1505 patients from three hospitals in Hong Kong and South Korea tertiary referral clinics. The name was the abbreviation of “Risk Estimation for HCC in CHB”. All patients in the training

**Table 2 Components of the risk scores**

Factor	Risk score			
	CU-HCC	GAG-HCC (yr)	REACH-B	
Age (yr)	≤ 50: 0		30-34: 0	
	>50: 3		35-39: 1	
			40-44: 2	
			45-49: 3	
			50-54: 4	
			55-59: 5	
Sex	NA	Male: 16 Female: 0	Male: 2 Female: 0	
	Albumin (g/L)	≤ 35: 20	NA	NA
		> 35: 0		
Bilirubin (mmol/L)	≤ 18: 0	NA	NA	
	> 18: 1.5			
ALT (U/L)	NA	NA	< 15: 0	
			15-44: 1	
			≥ 45: 2	
HBeAg	NA	NA	Positive: 2 Negative: 0	
	HBV DNA (copies/mL)	< 4 log: 0	3 × in log	< 4 log: 0
4-6 log: 1		4-5 log: 3		
> 6 log: 4		5-6 log: 5		
(lack of maintained virologic suppression: 4)		≥ 6 log: 4		
		(lack of maintained virologic suppression: 4)		
Cirrhosis	Presence: 15 Absence: 0	Presence: 33 Absence: 0	NA	

ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; NA: Not applicable; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

cohort did not have cirrhosis according to ultrasonography at the time of recruitment, and remained treatment-naïve throughout the follow-up period which was as long as 12 years. In contrast, 18.4% (277/1505) of patients in the validation cohort had cirrhosis. The REACH-B score consists of 5 parameters: gender, age, ALT level, HBeAg status and HBV DNA level. The score ranges from 0 to 17 and is primarily designed for patients without cirrhosis. The authors did not categorize patients into different risk levels, instead the 3-, 5- and 10-year risk of HCC was determined for each particular risk score. The HCC risk ranged from 0% to 23.6% at 3 years, 0% to 47.4% at 5 years, and 0% to 81.6% at 10 years for patients with the lowest (0 point) and highest HCC risk (17 points), respectively. As the risk increased significantly starting at 8 points, it could be used as an arbitrary cutoff value to categorize patients into different level of risks.

## IMPACT OF ANTIVIRAL THERAPY ON RISK PREDICTION

Most of the patients involved in the development of the risk scores did not receive antiviral therapy. This raised a concern regarding their validity and applicability to patients receiving treatment. This is particularly relevant to

**Table 3 Comparison of the CU-hepatocellular carcinoma, GAG-hepatocellular carcinoma and REACH-B scores**

Score	Patients	Components	Cutoff value	Performance
CU-HCC	Clinic patients: 1005 in training cohort, 424 in validation cohort	Age, albumin, bilirubin, HBV DNA, cirrhosis	5	97% NPV at 10 yr
GAG-HCC	820 clinic patients (leave-one-out cross-validation method)	Age, gender, HBV DNA, cirrhosis	101	99% NPV at 10 yr
REACH-B	Non-cirrhotic patients: 3584 in training cohort, 1505 in validation cohort	Age, gender, ALT, HBV DNA, HBeAg	8	98% NPV at 10 yr

ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; NPV: Negative predictive value.

those at risk of HCC as they most often receive antiviral therapy. Antiviral therapy modifies the natural history of CHB by decreasing the serum HBV DNA levels, and altering other laboratory parameters (*e.g.*, lowering ALT, raising albumin and lowering bilirubin level). This leads to another question on the clinical significance of dynamic changes in the risk scores during longitudinal follow-up.

These important concerns have been addressed in a recent cohort study of 1531 entecavir treatment CHB patients followed up for 42 ± 13 mo<sup>[22]</sup>. All patients received entecavir 0.5 mg daily for at least 12 mo. The importance of maintained viral suppression was emphasized in this study as virologic remission for 24 mo or more, together with advanced age and cirrhosis, were independent factors associated with HCC in this cohort. The CU-HCC, GAG-HCC and REACH-B scores were found to be accurate in predicting HCC in 3 and 5 years. Of these scores, the CU-HCC score had the highest AU-ROC at baseline (0.80 *vs* 0.76 and 0.71, respectively). At the recommended cutoff values, baseline CU-HCC and REACH-B scores had high sensitivity (93.6% and 95.2%, respectively), while the GAG-HCC score had high specificity (78.9%) in predicting HCC.

After antiviral therapy, the risk scores change due to decreased viral load (*i.e.*, lower HBV DNA) and even HBeAg-seroconversion, improvement in liver function (high albumin, lower bilirubin) and necroinflammation (lower ALT). Therefore, a significant proportion of patients would have decreased risk scores following treatment. From this cohort study, 14.0%, 8.2% and 38.3% of patients had their risk category changed from high risk to low risk as defined by the CU-HCC, GAG-HCC and REACH-B score, respectively, after 2 years of entecavir<sup>[22]</sup>. One unresolved issue is the regression of cirrhosis, which may occur after long-term antiviral therapy<sup>[28,29]</sup>. However, as this regression take years to happen, its effect on the dynamic change in risk level can only be evaluated in a study with at least 8 to 10 years of follow up.

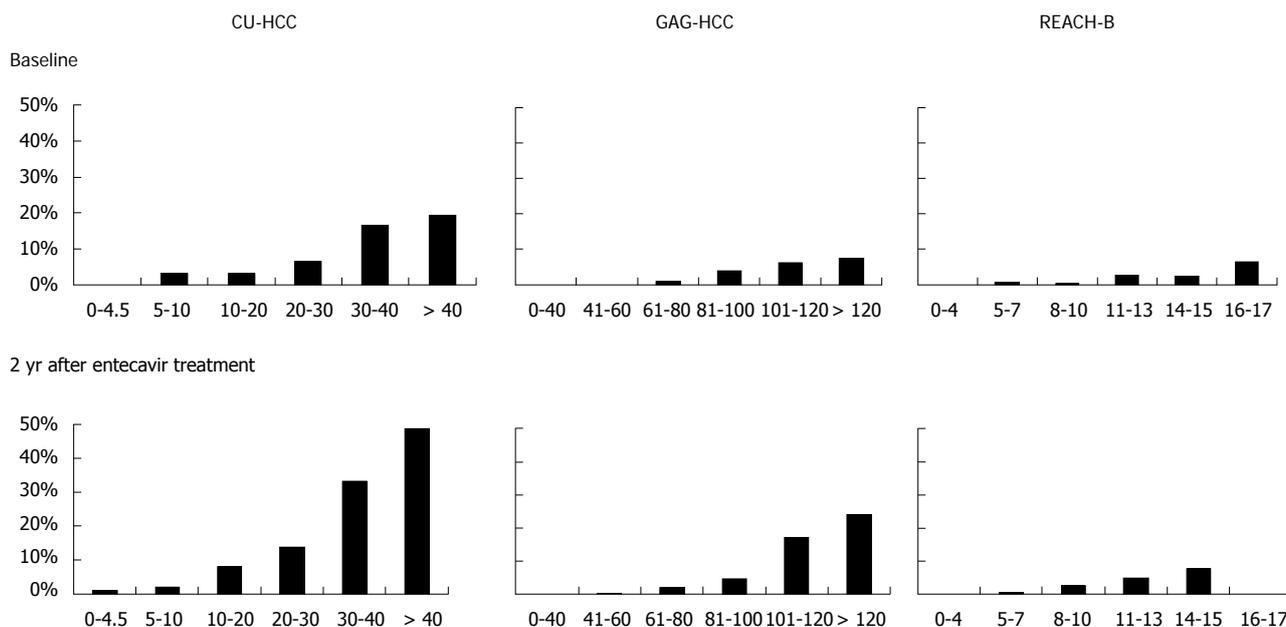


Figure 1 Risk of hepatocellular carcinoma in the next 3 years by risk scores. Results adopted from Wong *et al*<sup>[22]</sup>. HCC: Hepatocellular carcinoma.

**Table 4** Dynamic changes in risk scores and 5-year risk of hepatocellular carcinoma

Risk score	Baseline	2 yr	HCC in 5 yr		
			CU-HCC	GAG-HCC	REACH-B <sup>1</sup>
Low	Low	Low	0.4%	1.4%	0.0%
Low	Low	High	0.0%	NA	0.0%
High	High	Low	2.1%	5.1%	0.0%
High	High	High	12.9%	26.4%	2.1%

<sup>1</sup>Only patients without cirrhosis were analyzed for the REACH-B score. Results adopted from Wong *et al*<sup>[22]</sup>. HCC: Hepatocellular carcinoma; NA: Not available.

The dynamic changes in risk scores after antiviral therapy had a significant meaning on HCC risk. For all three risk scores, patients persistently in the low-risk category had the lowest risk of HCC; those “downgraded” in risk category had a significantly lower risk of HCC compared to those in the high-risk category (Table 4)<sup>[22]</sup>. Only 0.4% of patients who remained at low risk at baseline and 2 years according to the CU-HCC score would develop HCC in 5 years; the corresponding figures were 2.1% and 12.9% in those who changed from high risk to low risk, and those who remained at high risk at both time points, respectively. With the GAG-HCC score, 1.4%, 5.1% and 26.4% of patients who remained at low risk, changed from high to low risk, and remained at high risk developed HCC in 5 years, respectively. The results from both the CU-HCC and GAG-HCC score showed that downgrading of risk score reduces, but does not eliminate the risk of HCC (Figure 1).

## CLINICAL APPLICATION OF RISK SCORES

The risk scores discussed above are simple to use as they combine a few widely available clinical variables for the estimation of HCC risk within a specific timeframe. However, the version of the GAG-HCC score which includes core promoter mutations as a component may not be preferred by clinicians, as tests for these mutations are not easily accessible in the primary care setting and general practitioners taking care of the majority of CHB patients. The simple calculations in these scores facilitate implementation in routine clinical use. However, the complexity of these calculations is less of a concern as web-based or smart phone apps which include calculators for some of these scores are now available<sup>[30,31]</sup>. The major limitation of these scores is that all studies only involve Asian (mostly Chinese) patients, therefore, the validity and applicability in other ethnic groups remain uncertain. These risk scores can potentially be incorporated into a clinical risk-prediction instrument that could improve patient management through appropriate and timely intervention. Clinicians could use the scores to assess the risk of progression, and subsequently make evidence-based decisions about the clinical management of these patients. A recent Japanese study showed that patients in the high-risk categories according to these risk scores would benefit most from entecavir<sup>[5]</sup>. Another long-term follow-up study of 641 patients receiving tenofovir for 6 years showed that the observed incidence of HCC was lowered compared to the predicted risk us-

ing the REACH-B score<sup>[32]</sup>. This is indirect evidence that antiviral therapy reduces the risk of HCC.

We advocate estimating the risk scores for all CHB patients. For treatment-naïve patients, the results of these scores may guide the need for antiviral therapy complementary to the treatment guidelines. The scores should be monitored regularly every 1 to 2 years. Patients remaining at low risk are suitable for regular monitoring in the primary care setting. Those at high risk should be referred for specialist care and appropriate treatment should be considered.

For patients receiving antiviral therapy, the risk scores should be monitored yearly. Those who respond well to treatment, *i.e.*, achieve maintained virologic remission, and remain in the low-risk category have a minimal risk of HCC. Therefore, they may also be referred to family physicians who are experienced in monitoring such patients. Patients with risk downgraded after treatment would have a lower, but 2% to 5% risk of HCC in 5 years. Therefore, they should undergo regular HCC surveillance<sup>[33]</sup>. Those in the high-risk category despite antiviral therapy may require more intensive HCC surveillance, as the risk of HCC can be as high as 12.9% to 26.4% in 5 years (Table 3). On the other hand, patients who fail to achieve maintained viral suppression should consider alternative treatment regimes in order to reduce the risk of HCC<sup>[34]</sup>.

## FUTURE DIRECTION

One potential problem with these risk scores is that heavy weighting is assigned to cirrhosis in CU-HCC and GAG-HCC. In the study of the REACH-B score, liver cirrhosis was excluded by ultrasonography. As early cirrhosis may be missed by ultrasonography, this limitation may lead to substantial prediction errors if the presence or absence of cirrhosis is misclassified<sup>[35]</sup>. Transient elastography is one of the most widely validated non-invasive tools to detect early liver cirrhosis in various chronic liver diseases<sup>[36]</sup>. Liver stiffness measurement (LSM) with this tool may be useful to refine the diagnosis of cirrhosis and substitute clinical cirrhosis as a component in the risk score to predict HCC. There is evidence that LSM can predict HCC<sup>[37]</sup>, patient survival<sup>[38]</sup> as well as complications after hepatic resection<sup>[39]</sup>. Therefore, it is reasonable to believe that LSM would be an important parameter in a HCC risk score.

A recent Korean study of 1250 CHB patients developed a predictive model for HCC using four clinical parameters, which included age, gender, HBV DNA and LSM value<sup>[40]</sup>. The probability equals  $1 - P^A$ ; where  $A = \exp(0.05306 \times \text{age} + 1.106 \times \text{male gender} + 0.04858 \times \text{LSM values} + 0.50969 \times \text{HBV DNA} \geq 20000 \text{ IU/L})$ . This model was found to have a moderately good discrimination capability, with an AUROC of 0.81. The predicted risk of HCC development correlated fairly well with the observed risk ( $r = 0.91$ ). More data concerning the role of LSM in the HCC risk score is now evolving<sup>[41]</sup>.

## CONCLUSION

In conclusion, HCC risk scores can accurately predict subsequent HCC development in both treatment-naïve patients and in those receiving antiviral therapy. Different levels of care and different intensities of HCC surveillance should be offered according to the risk profile of patients. Patients in the high-risk category should be one of the indications for antiviral therapy, as well as appropriate HCC surveillance. For patients receiving antiviral therapy, maintained virologic response should be the treatment target, particularly in patients with cirrhosis. Patients at risk of HCC should undergo regular HCC surveillance, even when they are receiving antiviral treatment.

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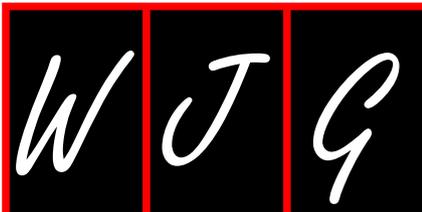
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## Gastroesophageal reflux disease: Update on inflammation and symptom perception

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### Abstract

Although gastroesophageal reflux disease (GERD) is a common disorder in Western countries, with a significant impact on quality of life and healthcare costs, the mechanisms involved in the pathogenesis of symptoms remain to be fully elucidated. GERD symptoms and complications may result from a multifactorial mechanism, in which acid and acid-pepsin are the important noxious factors involved. Prolonged contact of the esophageal mucosa with the refluxed content, probably caused by a defective anti-reflux barrier and luminal clearance mechanisms, would appear to be responsible for macroscopically detectable injury to the esophageal squamous epithelium. Receptors on acid-sensitive nerve endings may play a role in nociception and esophageal sensitivity, as suggested in animal models of chronic acid exposure. Meanwhile, specific cytokine and chemokine profiles would appear to underlie the various esophageal phenotypes of GERD, explaining, in part, the genesis of esophagitis in a subset of patients. Despite these findings, which show a significant production of inflammatory mediators and neurotransmitters

in the pathogenesis of GERD, the relationship between the hypersensitivity and esophageal inflammation is not clear. Moreover, the large majority of GERD patients (up to 70%) do not develop esophageal erosions, a variant of the condition called non-erosive reflux disease. This summary aims to explore the inflammatory pathway involved in GERD pathogenesis, to better understand the possible distinction between erosive and non-erosive reflux disease patients and to provide new therapeutic approaches.

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**Key words:** Gastroesophageal reflux disease; Mucosal inflammation; Heartburn; Esophagitis; Hypersensitivity

**Core tip:** The present study aimed to explore the mechanisms involved in the pathogenesis of gastroesophageal reflux disease (GERD) symptoms and complications, which remain to be fully elucidated. Despite recent evidence confirming the important production of inflammatory mediators and neurotransmitters in the pathogenesis of GERD, the interplay between esophageal inflammation and hypersensitivity is not clear. Based on the literature and on personal experimental studies, this paper attempts to summarize the evidence concerning the inflammatory pathway involved in GERD pathogenesis, to better define the possible distinction between erosive and non-erosive reflux disease patients and to provide new therapeutic approaches.

Altomare A, Guarino MPL, Cocca S, Emerenziani S, Cicala M. Gastroesophageal reflux disease: Update on inflammation and symptom perception. *World J Gastroenterol* 2013; 19(39): 6523-6528 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6523.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6523>

## INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder in Western countries, with 10%-30% of the individuals affected every week by symptoms that have a significant impact on quality of life and healthcare costs<sup>[1-3]</sup>. In GERD, the mechanisms involved in the pathogenesis of heartburn and chest pain remain to be completely elucidated; however, acid and pepsin can induce macroscopically detectable injury to the esophageal squamous epithelium<sup>[4]</sup>. Injured or inflamed tissues release inflammatory mediators that can be detected by the immune system. Moreover, in response to these chemical insults, endothelial cells produce adhesion molecules, which recruit and activate leukocytes, thus mediating inflammatory conditions<sup>[5]</sup>. In an experimental model of reflux esophagitis and in patients affected by GERD, some of the mediators considered to be critical in the etiology of esophagitis are classic inflammatory products, such as prostanoids and reactive oxygen species (ROS)<sup>[6,7]</sup>. Several studies have also suggested that the mucosal immune and inflammatory responses, characterized by specific cytokine and chemokine profiles, may underlie the various esophageal phenotypes of GERD<sup>[8-11]</sup>. Indeed, the large majority of GERD patients (up to 70%) do not develop esophageal erosions, a variant of the condition called non-erosive reflux disease (NERD)<sup>[12,13]</sup>. Although several studies report the progression of NERD to erosive esophagitis (erosive reflux disease, ERD), structural and functional characteristics differentiate these two important GERD groups: NERD patients usually have a normal lower esophageal sphincter resting pressure, minimal esophageal body motility abnormalities, low esophageal acid exposure profile and minimal night-time esophageal acid exposure<sup>[14,15]</sup>. The symptom response, in NERD patients, to acid suppressive therapy is lower than that in patients with ERD<sup>[14]</sup>. In fact, in these patients, esophageal visceral hypersensitivity, sustained esophageal contractions or impaired tissue resistance, have been identified as possible mechanisms responsible for reflux symptoms and proton pump inhibitor (PPI) resistance<sup>[16-18]</sup>.

This brief summary focuses on the inflammatory pathway involved in the pathogenesis of GERD, to better understand the distinction between ERD and NERD patients and, thus, to provide better therapeutic approaches.

## DAMAGE INDUCED BY REFLUXATE

GERD is a complex disorder with the potential for developing esophagitis, esophageal strictures and Barrett's esophagus<sup>[2]</sup>. GERD symptoms and complications may result from a multifactorial mechanism in which acid and acid-pepsin are the important noxious factors involved. Prolonged contact of the esophageal mucosa with the refluxed content, possibly caused by a defective anti-reflux barrier and luminal clearance mechanisms, would appear to be responsible for the morphological changes in the esophageal epithelium of GERD patients<sup>[19,20]</sup>.

A well studied ultra-structural alteration, *i.e.*, dilated intercellular spaces (DIS), demonstrated both in ERD and NERD patients<sup>[21,22]</sup>, could explain the genesis of symptoms triggered by the activation of intra-mucosal chemo-sensitive nociceptors and, at the same time, the inflammatory cascade generated by luminal acid diffusing into the tissue. In conditions not associated with severe inflammation, it is unclear how, in the presence of these symptoms, an injurious process initiating in the normal mucosa may lead to macroscopic injuries in a subset of patients (30%-40%), namely the ERD group.

The increased paracellular permeability, associated with the presence of DIS, and the resulting breakdown in the epithelial barrier, do not necessarily result only from excessive acid exposure, as suggested by the normal acid contact time at pH-monitoring in NERD patients, in which the symptoms are generated in the absence of esophageal epithelial erosions<sup>[23]</sup>. At the same time, the esophageal mucosa would appear to play a pivotal role in the development of esophageal inflammation and pain. Unlike intestinal inflammation, in which the role of the mucosa has been studied, as far as concerns the esophagus, it has only recently been suggested that gastric juice reflux does not directly damage the esophageal mucosa, but instead stimulates the esophageal epithelial cells to secrete chemokines that attract and activate the immune cells, causing damage to the esophageal squamous epithelial cells<sup>[23]</sup>. Microscopic inflammation, characterized by neutrophilic and eosinophilic infiltration of the esophageal mucosa and submucosa, is observed more frequently in ERD than in NERD patients<sup>[24-26]</sup>.

Therefore, the different esophageal phenotypes of GERD could possibly reflect the presence of various inflammatory mediators responsible for mucosal immune responses in these groups of patients<sup>[8,27,28]</sup>.

In a recent investigation, we observed that levels of cytokines, such as interleukin-8 (IL-8) and platelet activating factor (PAF), are significantly higher in the esophageal mucosa of ERD patients compared with those in the NERD group, in whom the expression of these inflammatory mediators is comparable to those of controls<sup>[11]</sup>. The acid-induced production of IL-8 and other inflammatory mediators by the esophageal mucosa have been shown to promote migration and activation of peripheral blood leukocytes<sup>[29]</sup>. These findings would corroborate the hypothesis that a cytokine-mediated mechanism, rather than a direct effect of gastroesophageal reflux, is responsible for the mucosal injury in the ERD subgroup.

Growing evidence shows that NERD patients are characterized by enhanced esophageal sensitivity to chemical and mechanical stimuli caused by enhanced excitability of visceral sensory neurons, possibly associated with overexpression of acid-sensing receptors in the epithelial layer and in the afferent fibers of the *lamina propria*<sup>[30,31]</sup>.

In particular, the transient receptor potential channel vanilloid subfamily member-1 (TRPV1) is overexpressed in the esophageal mucosa of ERD and NERD patients,

**Table 1** Cytokines and Chemokines involved in the pathophysiology of gastroesophageal reflux disease

Patients	Cytokines	Chemokines
GERD	IL-6 <sup>[8,23]</sup> , and IL-8 <sup>[8-11,23,25,29]</sup> , IL-1 $\beta$ <sup>[23,28,32,38]</sup> , INF- $\gamma$ <sup>[8]</sup> , TNF- $\alpha$ <sup>[20,38]</sup>	
ERD	IL-6 <sup>[32,37]</sup> , IL-8 <sup>[8-11,25,28]</sup> , IL-1 $\beta$ <sup>[28,32,37,38]</sup>	PAF <sup>[11,36,37]</sup> , MCP-1 <sup>[9-11,36,38]</sup> , RANTES <sup>[9,36]</sup> , MIP-1 $\alpha$ <sup>[11,36,38]</sup> , Eotaxin-1, Eotaxin-2 and Eotaxin-3 <sup>[11,36]</sup> , CINC-2 $\alpha$ <sup>[38]</sup> , and ICAM-1 <sup>[38]</sup>
NERD	IL-8 <sup>[10,25,28]</sup> , IL-1 $\beta$ <sup>[28]</sup>	
Barrett's esophagus	IL-8 <sup>[8]</sup> , IL-4 <sup>[8]</sup> , TNF- $\alpha$ <sup>[34]</sup> , IL-6 <sup>[34]</sup> , IL-8 <sup>[35]</sup>	

GERD: Gastroesophageal reflux disease; ERD: Erosive reflux disease; NERD: Non-erosive reflux disease; IL-6: Interleukin-6; IL-8: Interleukin-8; IL-1 $\beta$ : Interleukin-1 $\beta$ ; INF- $\gamma$ : Interferon-gamma; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; PAF: Platelet-activating factor; MCP-1: Monocyte chemoattractant protein; MIP-1 $\alpha$ : Macrophage inflammatory protein-1 $\alpha$ ; CINC-2 $\alpha$ : Cytokine-induced neutrophil chemoattractant-2 $\alpha$ ; ICAM-1: Intercellular Adhesion Molecule-1; RANTES: Regulated upon activation normal T cell expressed and presumably secreted.

compared with healthy controls, which may explain the similar severity of reflux symptoms in both groups, regardless of the presence or absence of inflammation and erosions<sup>[25]</sup>.

## INFLAMMATORY MEDIATORS AND GERD PATHOGENESIS

The mucosa of GERD patients produces significantly larger amounts of various cytokines compared with that of healthy controls<sup>[8-10,26,28,32]</sup>. These inflammatory mediators activate immune cell recruitment and migration, and may be involved in the pathophysiology of GERD (Table 1).

IL-8, one of the most important neutrophil chemoattractants<sup>[33]</sup>, is overexpressed in the mucosa of GERD patients<sup>[8-10]</sup>, and increased IL-8 levels in the esophageal mucosa of these patients correlate with the endoscopic and histological severity of the disease<sup>[8-10]</sup>. Moreover, IL-8 levels decrease following PPIs<sup>[34]</sup> and following anti-reflux surgery<sup>[35]</sup>, possibly indicating a role of this chemokine in mucosal damage. Acid-induced production of IL-8 and PAF by the esophageal mucosa promotes the migration of peripheral blood leukocytes. PAF also induces the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by peripheral blood leukocytes<sup>[29]</sup>.

In a previous study, we have shown that acid-induced inflammation of the esophagus begins with activation of the TRPV1 receptors in the mucosa and synthesis of PAF by the epithelial cells<sup>[36]</sup>. Furthermore, the sensory neurotransmitters, calcitonin-gene related peptide (CGRP) and substance P, are produced by sensory neurons located in the esophageal mucosal layer<sup>[29]</sup>.

PAF diffuses from the mucosal layer, stimulating the production of H<sub>2</sub>O<sub>2</sub> in leukocytes and the production of IL-6 in the circular muscle, where IL-6 causes production of additional H<sub>2</sub>O<sub>2</sub> through activation of NADPH oxidases present in the circular muscle layer<sup>[37]</sup>. In turn, H<sub>2</sub>O<sub>2</sub> triggers the formation of IL-1 $\beta$ , which may induce the production of PAF in the muscle, probably supporting a self-sustaining cycle of inflammatory mediator production. Indeed, in an animal model of chronic esophagitis, significantly increased expressions of other inflammatory mediators, among which IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1),

macrophage inflammatory protein-1  $\alpha$  (MIP-1 $\alpha$ ) and eotaxins, were detected in esophageal lesions compared with the normal esophagus<sup>[11,38]</sup>. In agreement with these previous reports, we recently confirmed, in biopsies from ERD patients, a significant increase in the expression of IL-8, PAF and several chemokines, compared with controls<sup>[25]</sup>. Interestingly, unlike ERD patients, the esophageal mucosa of NERD patients did not exhibit enhanced expressions of various inflammatory mediators, or the significant presence of neutrophils and eosinophils in the mucosa, being comparable to asymptomatic controls<sup>[11,23,24]</sup>.

This hypothesis is supported by a multicenter, randomized, controlled trial including 514 patients affected by GERD<sup>[26]</sup>. The study revealed that “microscopic esophagitis” (dilatation of intercellular spaces, papillary elongation and basal cell hyperplasia), was found in more than 90% of ERD patients, but in only approximately 2/3 of NERD patients<sup>[26]</sup>, with significant infiltration of immune cells only in the ERD group.

These findings would indicate a key role of several soluble mediators acting as powerful inflammatory activators, contributing to the induction of esophagitis. The observation that CGRP and substance P are generated by different mechanisms and that two different pathways mediate the sensation of heartburn and inflammation, would further explain the presence of recurrent and severe symptoms, irrespective of mucosal injury.

## VISCERAL HYPERSENSITIVITY AND REFLUX PERCEPTIONS IN GERD PATIENTS

The pathogenesis of heartburn and acid regurgitation remain to be fully elucidated, particularly in the numerous NERD patients in whom the 24-h pH-test findings may be within the normal range<sup>[39]</sup>. Indeed, an enhanced sensitivity to reflux would appear to be strongly associated with the failure of PPI treatment<sup>[40]</sup>.

Although gastric acid plays a pivotal role in the pathogenesis of GERD, other stimuli have been suggested to be involved in the pathogenesis of typical symptoms. In patients with GERD, reflux may result in direct activation of pain receptors, which may be enhanced by greater acid

diffusion through the esophageal epithelium caused by impaired mucosal barrier function<sup>[19]</sup>. Furthermore, activation of pain receptors may occur following reflux-induced distention of the esophagus. Enhanced esophageal sensitivity to these stimuli may be caused by upregulation of peripheral pain receptors and central sensitization of spinal neurons<sup>[19]</sup>.

Little is known about acid-sensitive nerves. Receptors on acid-sensitive nerve endings may play a role in nociception and esophageal sensitivity, as suggested in animal models following chronic acid exposure, and include the anion-sensing ion channel (ASIC), TRPV1 and ionotropic purinergic (P2X and P2Y) receptors<sup>[4]</sup>.

The recently demonstrated presence of acid-sensitive TRPV1 receptors in sensory nerve fibers and in epithelial cells of the esophageal mucosa<sup>[41]</sup> provides an interesting mechanism to better understand the onset of neuromodulation and symptoms generation in GERD. TRPV1 activation in primary afferent neurons evokes the sensation of burning pain and may induce neurogenic inflammation following the release of substance P and CGRP<sup>[36]</sup>.

On the other hand, growing evidence from animal models during chronic acid exposure supports the involvement of purinergic receptors in nociception and hypersensitivity<sup>[38,39]</sup>. The purinergic receptors are involved in several cell functions and may be activated by purine nucleotides as ligands<sup>[42]</sup>.

Based on their pharmacological properties and molecular characteristics, two distinct classes of purinergic receptors with preferential responses to adenosine 5'-triphosphate (ATP), as well as other single nucleotides, have been identified: the family of ligand-gated cation channel P2X receptors and the G protein-coupled P2Y receptors.

P2X and P2Y receptors play an important role in signaling visceral pain<sup>[19,39,42]</sup>. A working hypothesis of purine-mediated mechano-sensory transduction has been suggested<sup>[19,39]</sup>: ATP released from the epithelial cell lining of the gastrointestinal (GI) tract, bladder and ureter might activate P2X receptors present on the sub-epithelial nerve plexus and the signal is transmitted *via* the spinal cord to the brain.

To date, a limited number of studies have been performed on changes in purinergic signaling in GI disorders. Extracellular nucleotides and their receptors have been implicated in the pathogenesis of various pathological conditions in the gut; indeed, adenosine increases vagal afferent sensitization in the esophagus and is able to activate a different type of nociceptive nerve terminal in this tissue<sup>[43]</sup>. Acid sensitizes P2X receptors to ATP, and acid-induced upregulation and activation of P2X receptors has been confirmed in animal models of esophagitis<sup>[44,45]</sup>.

However, the role of purinergic receptors in patients with GERD remains to be fully determined. In a recent investigation, we studied a signaling pathway that might be responsible for esophageal nociception, which involves ATP and purinoceptors. In an experimental model of acid-induced activation of the esophageal mucosal

nociceptors, we observed that acid exposure caused activation of TRPV1 receptors on the esophageal epithelial cells, triggering production of ATP, which acts on peripheral nerve terminals inducing the production of neurotransmitters<sup>[46]</sup>. Thus, the selective presence of purinergic receptors on esophageal epithelial cells was demonstrated, suggesting a direct effect of the acid on the epithelium and a possible autocrine effect of ATP on these cells<sup>[19]</sup>. In fact, P2X4, P2X5 and P2Y14 receptors are expressed in esophageal epithelial cells, and indeed are expressed at higher levels than all the other purinergic receptors<sup>[46]</sup>. P2Y receptors appear to be more involved in esophageal motility. Lecca *et al*<sup>[47]</sup> recently reported that purinoceptors are involved in human lower esophageal sphincter (LES) relaxation, mediated by neural nitric oxide and partially by P2Y receptors. Blockade of P2Y receptors reduced the amplitude of contractions without affecting the latency. Farrè *et al*<sup>[48]</sup> had previously demonstrated, in animals, that LES relaxation, induced by stimulation of the inhibitory motor neurons of the mesenteric plexus, was mediated by neural nitric oxide with a minor contribution of purines, acting by way of P2Y and P2X receptors.

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## CONCLUSION

Inflammatory processes in the esophageal mucosa appear to be involved not only in the development of erosive disease, but also in the early and important phases leading to hypersensitivity to intra-luminal stimuli.

Despite considerable evidence reconfirming the important production of inflammatory mediators and/or neurotransmitters in the pathogenesis of GERD, the interplay between hypersensitivity and esophageal inflammation remains unclear. Moreover, in the pathogenesis of GERD and in the generation of symptoms, receptors on acid-sensitive nerve endings may play a significant role. Further studies are warranted to better understand the signaling pathway involved in the genesis of reflux symptoms and inflammation, to identify and establish new therapeutic approaches.

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## Proton pump inhibitor resistance, the real challenge in gastro-esophageal reflux disease

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### Abstract

Gastro-esophageal reflux disease (GERD) is one of the most prevalent chronic diseases. Although proton pump inhibitors (PPIs) represent the mainstay of treatment both for healing erosive esophagitis and for symptom relief, several studies have shown that up to 40% of GERD patients reported either partial or complete lack of response of their symptoms to a standard PPI dose once daily. Several mechanisms have been proposed as involved in PPIs resistance, including ineffective control of gastric acid secretion, esophageal hypersensitivity, ultrastructural and functional changes in the esophageal epithelium. The diagnostic evaluation of a refractory GERD patients should include an accurate clinical evaluation, upper endoscopy, esophageal manometry and ambulatory pH-impedance monitoring, which allows to discriminate non-erosive reflux disease patients from those presenting esophageal hypersensitivity or functional heartburn. Treatment has been primarily based on doubling the PPI dose or switching to another PPI. Patients with proven disease, not responding to PPI twice daily, are eligible for anti-reflux surgery.

**Key words:** Gastro-esophageal reflux disease; Proton pump inhibitor; Ambulatory pH-impedance monitoring; Esophageal hypersensitivity; Gastro-esophageal reflux disease treatment

**Core tip:** The present review focuses on the subgroup of patients in whom proton pump inhibitor refractoriness more frequently occurs, on the mechanisms possibly involved in the lack of response, the diagnostic work-up and the therapeutic strategies in these patients. Various mechanisms and factors have been demonstrated and some mechanisms have also been proposed, although not yet supported by strong evidence. In the management of these patients, a careful clinical interview might conduct the diagnostic evaluation and the therapeutic approaches.

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### INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most prevalent chronic diseases in Western countries, affecting approximately 20% of the United States adult population weekly, and 7% daily<sup>[1,2]</sup>. Although the acid-suppressive drugs have improved in efficacy over the last few decades, and proton pump inhibitors (PPIs) represent the mainstay of treatment both for healing erosive esophagitis and for symptom relief as well as for preventing complications, several studies have shown that up to 40% of GERD patients reported either partial or complete lack of response of their symptoms to a

standard PPI dose once daily<sup>[3-5]</sup>. Therefore, particularly in third referral Gastrointestinal Units, the management of refractory GERD patients is a very common, as well as a very challenging, task. Indeed, chronic heartburn is associated not only with a significant decrease in all the physical and mental domains of health-related quality of life questionnaires but, also, with a significant increase in healthcare costs, due to repeated diagnostic procedures, physician examinations and drug prescriptions<sup>[6]</sup>. The present review focuses on the subgroup of patients in whom PPI refractoriness more frequently occurs, on the mechanisms possibly involved in the lack of response, the diagnostic work-up and the therapeutic strategies adopted in these patients.

## MOST DIFFICULT PATIENTS

The clinical suspicion that the symptomatic response to PPIs is less frequent in those patients affected by the most common presentation of GERD, *i.e.*, non-erosive reflux disease (NERD), than in those presenting erosive esophagitis (ERD) has been confirmed several years ago. In one of the first reports focusing on NERD patients, treatment with omeprazole 20 mg for 4 wk resulted in complete symptom relief in only 46% of patients, in even fewer of them on 10 mg and in those receiving placebo, and symptom improvement (satisfaction) in 66%<sup>[7]</sup>. The main messages of the study were the better results obtained with higher doses, which do not support the concept of NERD as a milder form of GERD and, more important, the concept that symptom relief proves to be directly correlated with esophageal acid exposure time, that is to say, the greater the acid exposure, the higher the PPI response. So far, only a few trials have compared the outcome of PPI treatment in NERD *vs* ERD patients. Almost all of these trials were carried out using a double blind, parallel group design with a short (4 wk) follow-up period. In a study performed by Bate *et al.*<sup>[8]</sup>, relief of heartburn was achieved in 47% of NERD, and in 53% of ERD patients (the difference not being significant). Of interest, as far as concerns the non-responders, 67% became heartburn-free after an additional 4 wk of treatment<sup>[8]</sup>.

Better results, both in NERD and ERD patients, have been reported in a multicenter study by Venables *et al.*<sup>[9]</sup>: heartburn relief, was achieved after 4 wk of omeprazole, in more than 60% of NERD and in 79% of ERD patients. Galmiche *et al.*<sup>[10]</sup>, besides heartburn remission, reported semi-quantitative measures of symptom severity and their impact on quality of life: At 4 wk, heartburn was resolved in 62% of NERD and 71% of the ERD patients, even higher values being observed after an additional 4-wk treatment with omeprazole. Of interest, quality of life improved in all treatment groups, but the improvement was higher in those on full PPI dose (*vs* half-dose) group<sup>[10]</sup>. Armstrong *et al.*<sup>[11]</sup>, in a randomized, Canadian multicenter study, confirmed complete relief in a larger proportion (although not significant) of ERD,

than NERD, patients receiving pantoprazole. Although some data were not stratified for the presence/absence of esophagitis, a modified intention-to-treat analysis demonstrated, in the PPI group, a trend of increased therapeutic gain throughout the 4 wk<sup>[11]</sup>. More recently, a multicenter trial performed in Japan, has shown that, following 4-wk rabeprazole 40 mg/die, complete relief of symptoms was achieved in only 36% of the NERD and in approximately 55% of the erosive group, a response rate similar to that observed in Western countries. Here, patients were stratified according to a modified Los Angeles classification and, of interest, the more severe the esophageal mucosal injury, the more effective the therapy. The design of the study and symptom assessment could also demonstrate that the median time to the first 24- and 48-h heartburn-free intervals was significantly shorter for erosive than for non-erosive patients<sup>[12]</sup>. Before concluding the issue regarding the response to PPI treatment in non-erosive *vs* erosive reflux disease, it may be useful to re-consider a major dilemma concerning NERD, namely the lack of a standard definition, which is likely to affect the results of clinical trials, and makes interpretation of data, challenging. It is generally agreed that NERD is the most common presentation (up to 75%) of GERD, with the same symptom severity and quality of life impairment as ERD, but, at the same time, there is still lack of agreement concerning the definition of NERD: should all symptomatic patients with endoscopy-negative findings be considered to be suffering from NERD? The 24-h pH test does, indeed, distinguish patients with and without pathological esophageal acid exposure, and, more important, patients with and without significant symptom-reflux association, which can reveal hypersensitivity to non-pathological acid exposure.

Endoscopy-negative patients not presenting pathological acid exposure, with negative symptom-reflux association and without a satisfactory response to the PPI test are, indeed, affected by functional heartburn, according to the Rome III criteria, and thus do not belong to the NERD population. These “functional” patients, in whom symptoms are, by definition, not related to reflux, might be a minority but they frequently attend the outpatients units and are, often, enrolled in clinical trials. The low response to PPIs reported in NERD may be affected by including this functional subgroup in a “too heterogeneous” NERD population. Another common risk of mis-classification of NERD is due to the healing of esophagitis at the time of upper endoscopy, and, thus, a recent consensus underlines the importance not only of an appropriate pharmacological washout before endoscopy but, also, of checking for previous endoscopic findings in the same patient, if available<sup>[13]</sup>. In the attempt to better evaluate the response rate in NERD patients according to the different criteria of the participants enrolled in clinical trials, a recent meta-analysis of the literature has demonstrated that lower rates of partial or complete response are reported in the large majority of studies with a poor characterization of the patients, lacking pH-test findings

**Table 1** Principal mechanisms and factors involved in proton pump inhibitor resistance

Adherence to PPI therapy
Compliance
Dosing, time
Reflux pattern
Weakly acidic reflux
Proximal reflux
Mixed reflux
Residual acid refluxes
Esophageal hypersensitivity
Other mechanisms
Reduced PPI bioavailability
Increased PPI metabolism
Mutations <i>cyt. p450</i>

PPI: Proton pump inhibitor.

and, therefore, likely including patients with functional heartburn and functional dyspepsia<sup>[14]</sup>. Future studies, enrolling well-defined NERD patients and, hopefully, with a longer follow-up, might offer more precise data on PPI efficacy.

## MECHANISMS AND FACTORS INVOLVED IN PPI RESISTANCE

In patients with reflux symptoms refractory to medical therapy, namely those with typical GERD symptoms - heartburn and regurgitation - not responding to a standard or double dose of PPI given for at least 8 wk, various causes have been demonstrated and some mechanisms have also been proposed, although not yet supported by strong evidence. Principal mechanisms and factors involved in PPI resistance are summarized in Table 1.

Ineffective control of gastric acid secretion, in terms of excessive residual acid reflux despite adequate PPI treatment, can be due to lack of compliance, rapid PPI metabolism - due to CYP2C19 polymorphism - or hypersecretory syndromes such as Zollinger Ellison. While these two latter conditions are uncommon, non-compliance to treatment, in terms of incorrect medication dose or timing, is reported to frequently occur. Two recent meta-analyses have clearly shown that lack or non-compliance to therapy is particularly frequent in GERD patients, in whom adherence to the prescribed PPI is acceptable in only 55% of patients, at one month, and in 30% at 6 mo after prescription.

The lowest levels of compliance, in terms of daily or dose administration, were observed in NERD patients, and, of the various factors, the most frequently reported were: lack of knowledge about the treated disorder, desire for personal control, side-effects and additional medications<sup>[15]</sup>. In a study focusing on patients with persistent GERD symptoms despite prolonged PPI treatment, it was reported that in less than 46% of these patients the drug was administered in the fasting state, before breakfast<sup>[16]</sup>.

In the new era of combined pH and impedance 24-h

monitoring, it is possible to detect reflux episodes with more accuracy compared to the pH-monitoring alone, following the movement of refluxate along the esophageal body and to distinguish air/liquid component as well as acidic composition of each episode. Over the last decade, several pH-impedance investigations have been conducted on patients with NERD and, particularly, on those patients with a poor symptomatic response to PPIs. Results emerging from those studies have confirmed a condition already observed with pH-tests, namely esophageal hypersensitivity in terms of perception of not-abnormal reflux, and this enhanced sensitivity involves not only acidic reflux but, also, weakly acidic reflux and gas-containing (mixed) reflux episodes. Either cohort studies analyzing the reflux pattern and reflux-symptom association<sup>[17]</sup> or pathophysiologic investigations, looking at the perception of each reflux episode<sup>[18]</sup> have clearly shown that, in NERD patients, besides acidic reflux, weakly acidic reflux and gas-containing episodes (both of them probably associated with increased reflux volume and esophageal distension) are responsible for a significant proportion of symptoms (approximately 20%), much higher when compared to those in ERD patients. These studies have demonstrated both a possible mechanism explaining symptom persistence despite acid suppression and the higher diagnostic yield of the pH-impedance test in these patients.

Recent pathophysiological investigations have also shown that a dynamic characteristic, such as the proximal migration of reflux, an indicator of high volume refluxate, represents a major determinant of reflux perception, particularly in NERD patients. Interestingly, in large multicenter studies, these three characteristics, namely weakly acidic reflux, mixed (liquid-gas) reflux and the higher proximal extent, have also been recognized as the main mechanisms underlying failure of PPI treatment in patients with reflux-related symptoms<sup>[19-21]</sup>. Finally, experimental studies suggest that some of the NERD patients presenting PPI-resistance may also present a more generalized condition of visceral hyperalgesia<sup>[22]</sup>.

The research field focusing on the ultrastructural and functional changes in the esophageal epithelium has contributed to a better understanding of NERD and of PPI-resistance pathophysiology. In those conditions not associated with severe mucosal inflammation and/or epithelial erosions, it is not clear how severe and recurrent symptoms can occur in an apparently normal mucosa (NERD). A well studied ultra-structural alteration, *i.e.*, dilated intercellular spaces (DIS), has been demonstrated by means of Transmission Electron Microscopy both in ERD and NERD patients<sup>[23,24]</sup>, and this would explain the genesis of symptoms triggered by the activation of intramucosal chemo-sensitive pain-receptors. The increased para-cellular permeability, associated with the presence of DIS, and the resulting breakdown in the epithelial barrier, do not necessarily result from excessive acid exposure, as shown in NERD patients presenting a normal acid contact time at pH-monitoring, can be induced, in ex-

perimental models, by weakly acidic and acidified bile solutions and even occurs during acute stress situations<sup>[25]</sup>. Interestingly, the feature of DIS has been observed in patients with PPI-resistant symptoms, during treatment, but not in patients affected by functional heartburn<sup>[26]</sup>, returns to normal following PPIs, together with symptoms<sup>[27]</sup>, and, therefore, the impaired mucosal integrity would now appear to be the mechanism that best explains the enhanced sensitivity to chemical and mechanical stimulation in NERD and PPI-resistant patients. Indeed, peripheral sensory pathways, in terms of up-regulated pain receptors, central sensitization of sensory neurons and processing of ascending stimuli are now under intense investigation and may be involved in the conditions of esophageal and visceral hypersensitivity.

Several conditions not, or not directly, related to gastro-esophageal reflux, should also be considered when assessing PPI refractoriness. Infectious esophagitis, eosinophilic esophagitis and pill esophagitis may be other, not frequent, causes of refractory heartburn. Anxiety and depression, demonstrated to increase reflux perception, may also be involved.

## DIAGNOSTIC EVALUATION

### *Clinical evaluation*

As previously pointed out, lack of compliance - in terms of adherence to treatment, timing and dosing - and the presence of functional heartburn are the main findings in patients referred for refractory heartburn, therefore a careful interview, also looking at the confounding presence/co-existence of atypical - ENT and respiratory - symptoms and at their possible relation with GER, is crucial. The presence of functional disorders, such as functional dyspepsia and irritable bowel syndrome, as well as of psychological disorders, should also be assessed as these are associated with visceral hyperalgesia as well as with reduced response to acid-suppressive drugs.

### *Endoscopy*

Although the sensitivity of upper endoscopy is very low - most patients have NERD - it might be helpful for ruling out pill and infectious esophagitis, eosinophilic esophagitis (4%-6% in PPI-refractory patients, multiple biopsies should be obtained) and the rare cases of Zollinger Elison syndrome.

### *Esophageal manometry*

Conventional or high-resolution manometry should be performed in order to rule out severe motor disorders, to better locate LES for pH-sensor positioning, and, furthermore can provide useful information when a surgical anti-reflux approach is indicated.

### *Ambulatory pH [impedance] monitoring*

The only test which provides quantitative information on the esophageal exposure to reflux, also assessing its

relationship with symptoms, remains 24-h ambulatory pH-monitoring. Prolonged (48 to 96 h) wireless pH-monitoring improves the diagnostic yield of the test by improving the likelihood of a positive reflux-symptom association<sup>[28]</sup>. We have previously discussed the advantages of the combined ambulatory pH-impedance test, as well as its greater accuracy in discriminating NERD patients from those presenting esophageal hypersensitivity or functional heartburn. Indeed, typical and atypical symptoms not responding to PPIs represent the main indication for performing ambulatory pH-impedance monitoring. The test performed "off" therapy can confirm or exclude a pathological gastro-esophageal reflux and, according to a recent investigation<sup>[29]</sup> offers the best chances to detect a positive association between symptoms and reflux episodes. Recent studies have shown that refractory patients studied "off" and "on" therapy are, indeed, characterized by an abnormal number of reflux events and a higher sensitivity to all types of reflux - acidic, weakly acidic, mixed and propagated<sup>[30,31]</sup>. On the other hand, performing the test "on" PPIs, provides useful information regarding the efficacy of acid-suppressive treatment and may detect a positive association between symptoms and weakly acidic reflux episodes - the large majority of episodes during acid suppressive drug -, which is a possible indication for anti-reflux surgery<sup>[32]</sup>.

## MANAGEMENT OF PATIENTS

### *Proton pump inhibitors*

The large majority of patients with reflux symptoms receive PPI therapy once daily. If symptoms are not relieved, and after the presence of functional heartburn and CYP2C19 polymorphism have been excluded, several therapeutic strategies can be proposed. These include doubling the current PPI dosage or switching to another PPI.

Indeed, treatment failure may result from an insufficient dose of PPI. Doubling the PPI dose, giving PPI before breakfast and before dinner, is one of the most common therapeutic strategies adopted by practicing physicians having also been recommended in the 2008 American Gastroenterological Association guidelines for GERD, and confirmed by the Cochrane review<sup>[33,34]</sup>. However, is still not clear the dose-response relationship for heartburn resolution in either erosive esophagitis or non-erosive reflux disease patients<sup>[35]</sup>. Even if doubling the PPI dose has become one of the standard strategies, escalation of the PPI administration beyond the twice daily dosage, both for symptom control or for healing of erosive esophagitis, is not supported by strong clinical data. In the attempt to identify the patients who would benefit from dose escalation, Becker *et al.*<sup>[36]</sup> performed pH-impedance monitoring in patients presenting persistent symptoms despite one month of standard PPI therapy. According to the pH-impedance data, two groups, one with and one without pathological findings, received high dose PPI (or fundoplication in a few cases). Imped-

ance was pathological in 40% of the non-responders, in whom escalating therapy was significantly more successful (90% relief) than in patients with normal findings.

Switching to another PPI is a very common, cost-effective, therapeutic strategy adopted in the management of patients who failed with the PPI once daily approach. In several studies, switching those patients who had failed with a PPI to esomeprazole, resulted in significant symptom improvement<sup>[37,38]</sup>.

### Antireflux surgery

Although it is well established that patients with symptoms not responding to PPIs have a less favorable post-operative clinical outcome compared to those patients responding to treatment, refractory GERD represents the most common (88%) indication for anti-reflux surgery. In a recent long-term follow-up study, 82% of the PPI-refractory patients reported that the preoperative reflux symptoms were completely resolved, and 94% were satisfied with the results of the surgery<sup>[39]</sup>. Several studies have suggested that a positive symptom-reflux association<sup>[40,41]</sup> and/or pathological AET<sup>[42,43]</sup>, observed by impedance-pH monitoring in patients off PPI, predict a favorable response to surgery. It has been recently demonstrated that ranitidine 300 mg twice daily has a comparable efficacy respect to rabeprazole 20 mg twice daily when given on-demand for the treatment of NERD and both medications are associated with improvement of the quality of life<sup>[44]</sup>.

It should be taken into consideration that the large majority of PPI-resistant patients do not present an erosive disease, therefore, given the possible adverse events associated with surgery and the recognized benign course of NERD, anti-reflux surgery should only be considered in selected patients, in whom objective evidence of reflux is revealed upon investigation. In summary, although surgery appears to be valid therapeutic option in GERD patients with typical symptoms who failed to respond to PPIs, further outcome and controlled studies, on a larger series of patients, using combined impedance-pH monitoring are warranted in order to draw definite conclusions.

### Lifestyle modifications

It has been recently suggested that weight loss and elevation of head of the bed are effective in improving GERD symptoms in refractory patients, whilst no sufficient data support any other lifestyle modifications<sup>[45]</sup>. It has been recently reported that shorter dinner-to-bed time interval (less than 3 h) is significantly associated with persistence of GERD symptoms<sup>[46]</sup>.

However, the relevance of lifestyle modifications in GERD patients who failed PPI treatment still remains to be fully elucidated.

### Visceral pain modulators, psychological treatment

The therapeutic option represented by visceral pain modulators is highly attractive but, at present, studies specifically evaluating their efficacy in refractory GERD

patients are still lacking. Tricyclic anti-depressants and selective serotonin reuptake inhibitors have been shown to relieve esophageal pain in patients with non-cardiac chest pain<sup>[46-48]</sup>. Unfortunately, side effects of these drugs appear to be not uncommon and may hamper their usage.

It has been shown that refractory patients are more likely to have a psychosocial comorbidity<sup>[49]</sup>, therefore it is conceivable that refractory GERD patients would benefit of psychological evaluation and treatment.

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Michele Cicala, MD, Professor, Series Editor

## Gastro-esophageal reflux disease and obesity, where is the link?

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**Key words:** Body mass index; Visceral obesity; Gastro-esophageal reflux; Gastro-esophageal reflux disease complication

**Core tip:** This topic is aimed to explore the potential mechanisms responsible for the association between gastro-esophageal reflux disease (GERD) and obesity, that remain to be fully elucidated. Despite numerous evidence that show an increased risk of GERD symptom and/or complication in obese patients, the interplay between GERD and obesity is not clear. Based on the literature we have tried to summarize the evidence concerning the role of obesity in the GERD pathogenesis to better understand the possible role of weight loss as a therapeutic approach for this disease.

### Abstract

The confluence between the increased prevalence of gastro-esophageal reflux disease (GERD) and of obesity has generated great interest in the association between these two conditions. Several studies have addressed the potential relationship between GERD and obesity, but the exact mechanism by which obesity causes reflux disease still remains to be clearly defined. A commonly suggested pathogenetic pathway is the increased abdominal pressure which relaxes the lower esophageal sphincter, thus exposing the esophageal mucosal to gastric content. Apart from the mechanical pressure, visceral fat is metabolically active and it has been strongly associated with serum levels of adipocytokines including interleukin-6 and tumor necrosis factor  $\alpha$ , which may play a role in GERD or consequent carcinogenesis. This summary is aimed to explore the potential mechanisms responsible for the association between GERD and obesity, and to better understand the possible role of weight loss as a therapeutic approach for GERD.

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### INTRODUCTION

Gastro-esophageal reflux disease (GERD), with symptoms demonstrated to impair quality of life (QoL), appears to show important variation in its prevalence. When defined as at least weekly heartburn and/or acid regurgitation, the prevalence in the Western world generally ranges between 10% and 20% whereas in Asia the prevalence is reported to be less than 5%<sup>[1]</sup>. Longitudinal studies have addressed several risk factors for GERD, and indeed obesity is indicated as a potential risk factor<sup>[2]</sup>. Interestingly, obesity, typically defined as a body mass index (BMI) of > 30, has risen to epidemic levels in several regions of North America, Europe and Asia<sup>[3]</sup>. The confluence between the increased prevalence of GERD, in

**Table 1 Proposed mechanisms by which abdominal obesity causes reflux**

Mechanical factors	Increased intra-gastric and gastro-esophageal pressure gradient Increased risk of Hiatal Hernia Increased sensitivity to distension-induced TLESR Decreased lower esophageal sphincter pressure
Humoral factors	Increased level of adipocytokines including interleukin 6 and tumor necrosis factor $\alpha$
Motility disorders	Delayed gastric emptying rate and delayed esophageal clearing time

TLESR: Transient lower esophageal sphincter relaxation.

terms of symptoms, erosive esophagitis, Barrett's esophagus (BE), esophageal adenocarcinoma and of obesity has generated great interest in the association between these two conditions and the potential mechanisms responsible for this association.

Several studies have focused on the potential relationship between GERD and obesity and results emerging from those investigations have shown that obesity is associated with an increased risk of both GERD symptoms and complication *i.e.*, erosive esophagitis, BE and esophageal adenocarcinoma as compared to individuals with a normal BMI<sup>[4]</sup>. There was also some degree of a dose-dependent relationship between BMI and these GERD-related disorders and, moreover, a recent large cohort focusing on adult females reported a possible dose-response increase in the risk of GERD with a higher BMI even if within the normal range<sup>[5]</sup>.

## PATHOGENESIS

Even if several mechanisms by which obesity causes reflux disease have been proposed (Table 1). The pathogenetic pathway commonly suggested is the increased abdominal pressure which relaxes the lower esophageal sphincter (LES), thus exposing the esophageal mucosal to the gastric content<sup>[6,7]</sup>. Results of 24-h pH-monitoring studies have shown that obesity is associated with a significant increase in the number of reflux episodes, as well as long-lasting reflux episodes and the time with pH < 4, especially in the post-prandial period<sup>[8]</sup>. This finding has been confirmed, in a more recent study, also by means of pH-impedance monitoring: not only the acid reflux but also the number of non acid reflux episodes increased significantly as BMI increased<sup>[9]</sup>. A recent study aimed to assess the pressure morphology and function of the esophago-gastric junction, by using the high resolution manometry methodology, reported that due to obesity the gastro-esophageal pressure gradients are altered in a way that would promote the retrograde flow of gastric content into the esophagus. Both the intra-gastric pressure and the gastro-esophageal pressure gradient were clearly correlated with both the BMI and waist circumference but, when these were simultaneously analysed in a regression model, the waist circumference was found to be independently associated with the different pressure

gradients, whereas the relationship between BMI and pressure became non-significant or considerably reduced. In addition to abnormal pressure gradients, high-resolution manometry also revealed that obesity was associated with hiatus hernia (HH). There was a significant correlation between BMI, waist circumference and axial separation of the intrinsic LES and crural diaphragm, and it was postulated that this was a manifestation of pressure stress due to the increased intra-gastric pressure<sup>[10]</sup>. In agreement with this finding, in a retrospective case-control study assessing BMI in relation to esophagitis and HH, it was found that obesity is strongly associated with the combined occurrence of esophagitis and HH<sup>[11]</sup>. It is widely recognized that HH has several pathophysiological implications in the pathogenesis of GERD: increased incidence of strain-induced reflux, reduced LES pressure, impaired esophageal acid clearance and increased sensitivity to distension-induced transient lower esophageal sphincter relaxation (TLESR)<sup>[12]</sup>. Among mechanisms responsible of reflux TLESR seems to play the most important role<sup>[13]</sup>. The reflux pattern after a standard meal, has been recently evaluated in obese and overweight patients by using a combined 2 h post-prandial esophageal manometry and pH monitoring<sup>[14]</sup>. The results of this study have shown that, during the post-prandial period, both the obese and overweight patients presented a substantial increase in the rate of TLESRs and in the proportion of TLESRs with acid reflux as compared to individuals with a normal BMI. Both BMI and waist circumference showed a significant positive correlation with TLESRs and there was a dose-effect relationship. Therefore, it would appear that a higher post-prandial intra-gastric pressure causes a more intense stimulation both on the stretch and tension mechanoreceptors in the proximal stomach, which leads to more postprandial TLESRs.

## ABDOMINAL OBESITY

Abdominal or visceral fat (VAT) is strongly different in respect to peripheral or subcutaneous fat (SCAT). For example, VAT is more metabolically active, is characterized by a higher number of immune and inflammatory cells, and is more insulin-resistant thus leading to an higher overall mortality in respect to SCAT<sup>[15]</sup>.

Abdominal obesity can better explain some of the epidemiological features of BE and esophageal adenocarcinoma. The distribution of body fat tends to be more visceral than truncal in high-risk groups for BE<sup>[16]</sup>. A recent study has shown that the abdominal diameter measured as the waist circumference is a risk factor for BE independently of BMI, while the association between BMI and BE disappeared after adjustment of the abdominal diameter<sup>[17]</sup>. These studies indicate that abdominal fat is the key factor linking obesity and BE. Apart from the mechanical pressure, visceral fat is metabolically active, and has been clearly associated with serum levels of adipo-cytokines including interleukin 6 and tumor ne-

cross factor  $\alpha$ , which may play a role in GERD and/or consequent carcinogenesis<sup>[18]</sup>. In fact, in a very recent study, a large amount of visceral abdominal fat in respect to subcutaneous fat was found to be associated with a significant increase in the risk of BE<sup>[19]</sup>.

## OUTCOME

Considering the dose-response relationship between obesity and occurrence of GERD and/or the resulting complications, an inverse relationship between weight loss and GERD symptoms would be expected. The prevalence of GERD symptoms in overweight and obese subjects as well as impact of weight loss on GERD symptoms has been assessed in a recent prospective study<sup>[20]</sup>. Weight loss strategies included dietary modifications, increased physical activity as well as behavioral changes. The results coming from the study showed that weight loss led to a significant improvement in GERD symptoms, thus establishing weight loss as an important modification of life style in the treatment of GERD. Moreover, weight loss over a 6-mo period, was associated with a reduction in GERD symptoms in 81% of the patients and with complete resolution in 65% of the patients. This finding provides support for recommending weight-loss in the primary treatment of overweight reflux patients, however this clinically important finding has unfortunately not been described so far. Further investigations on the long-term effect of weight loss on reflux occurrence and on the reduction in reflux symptoms are needed before any definitive conclusions can be drawn regarding eventual beneficial effects of weight loss.

## CONCLUSION

Obesity appears to be involved not only in the development of GERD symptoms but, also in the occurrence of GERD complication such as erosive esophagitis, Barrett's esophagus and esophageal adenocarcinoma.

Despite considerable evidence confirming the important role of increased esophago-gastric pressure gradient, and of production of inflammatory mediators by abdominal adipose tissue in the pathogenesis of GERD, the interplay between obesity and GERD is still not clear. Moreover, weight loss seems to reduce GERD symptoms but further studies are warranted to better understand the exact mechanism by which obesity causes reflux disease in order to identify and establish new therapeutic approaches.

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## Concepts of oxidative stress and antioxidant defense in Crohn's disease

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### Abstract

Oxygen free radical and lipid peroxides (oxidative stress) are highly reactive and represent very damaging compounds. Oxidative stress could be a major contributing factor to the tissue injury and fibrosis that characterize Crohn's disease. An imbalance between increased reactive oxygen species levels and decreased antioxidant defenses occurs in Crohn's patients. Decreased blood levels of vitamins C and E and decreased intestinal mucosal levels of CuZn superoxide dismutase, glutathione, vitamin A, C, E, and  $\beta$ -carotene have been reported for Crohn's patients. Increased levels of proinflammatory cytokines, such as interleukin-1 and -8 and tumor necrosis factor, have been detected in inflammatory bowel disease. Oxidative stress significantly increased the production of neutrophils, chemokines, and interleukin-8. These effects were inhibited by antioxidant vitamins and arachidonic acid metabolite inhibitors in human intestinal smooth muscle cells isolated from the bowels of Crohn's disease patients. The main pathological feature of Crohn's disease is an infiltration of polymorphonuclear neutrophils and mononuclear cells into the affected part of the intestine. Activated neutrophils produce noxious substances that cause inflammation and tissue injury. Due to the physiological and biochemical actions of reactive oxygen species and lipid peroxides, many of the clinical and pathophysiological

features of Crohn's disease might be explained by an imbalance of increased reactive oxygen species and a net decrease of antioxidant molecules. This review describes the general concepts of free radical, lipid peroxide and antioxidant activities and eventually illustrates their interferences in the development of Crohn's strictures.

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**Key words:** Crohn's disease; Reactive oxygen species; Antioxidant enzymes; Lipid peroxide

**Core tip:** Crohn's disease is associated with an imbalance, comprising increased reactive oxygen species (ROS) and decreased net antioxidant activity. A deficiency in antioxidant molecules could lead to increased levels of lipid peroxides or ROS, which could act locally or be secreted into the circulation to produce different systemic effects in the patient. Future research should address the question of whether ROS are involved in increasing the production of the different extracellular proteins by enhancing the transcriptions of certain genes using specific transcription factors.

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### INTRODUCTION

Crohn's disease (CD) is a debilitating illness of the bowel characterized by chronic inflammation of unknown etiology<sup>[1]</sup>. In the United States and Europe, the incidence ranges from 6-20 cases per 100000 population per year, and the prevalence, from 90-300 per 100000 population.

This disease most often occurs between the ages of 15 and 20, with a secondary peak between the ages of 55 and 60<sup>[2]</sup>. It can affect any level of the alimentary tract, but the terminal ileum and proximal colon are most frequently involved. A classical feature of CD is the sharp demarcation of the affected bowel segment from adjacent uninvolved segments<sup>[1]</sup>. This disease is characterized pathologically as a recurrent granulomatous inflammation of all layers of the bowel wall<sup>[2-4]</sup>. Patients usually suffer from abdominal pain, cachexia, anorexia, chronic diarrhea, intestinal obstruction, malnutrition, weight loss, and growth failure.

The precise etiology of CD remains unclear. It is thought that interactions among various factors, including genetic factors, host immune system and environmental factors (including diets and microbiological agents), play crucial roles in disturbing the intestinal homeostasis, leading to the dysregulated inflammatory responses of the gut. As inflammation is intimately related to the formation of reactive intermediates, including reactive oxygen species (ROS), oxidative stress has been proposed as a mechanism underlying the pathophysiology of CD. The main pathological feature of CD is an infiltration of polymorphonuclear neutrophils and mononuclear cells into the affected part of the intestine<sup>[5-10]</sup>. As the disease becomes more established, neutrophils infiltrate isolated crypts, forming abscesses in the affected mucosa and underlying tissue layers<sup>[1]</sup>. Neutrophils, like other leukocytes, produce noxious substances, such as ROS, tumor necrosis factor alpha (TNF $\alpha$ ), interleukin-1 (IL-1) and protease<sup>[11-14]</sup>. It has been shown that an imbalance in the levels of proinflammatory and anti-inflammatory cytokines occurs in CD<sup>[13,14]</sup>. Increased levels of proinflammatory cytokines, such as IL-1, IL-8 and TNF $\alpha$ , can be detected in CD.

Reactive oxygen species may play an important role in CD. As will be discussed in the following sections, the ROS generated from activated leukocytes or from other sources may affect various biological molecules, generating tissue damage that could have been prevented by dietary antioxidants. This review describes the general concepts of free radical, lipid peroxide and antioxidant activities and eventually illustrates their interference into the development of Crohn's strictures.

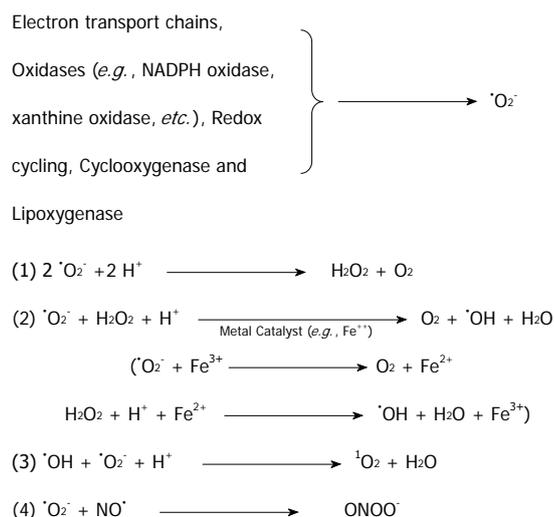
## CONCEPTS OF FREE RADICALS

A free radical can be defined as any chemical species that has one or more unpaired electrons in the outermost orbital electron shell<sup>[15]</sup>. Due to these chemical changes in the free radical atoms, they are chemically reactive. ROS include superoxide ( $\cdot\text{O}_2^-$ ), the hydroxyl radical ( $\cdot\text{OH}$ ), the hydroperoxyl radical ( $\cdot\text{O}_2\text{H}$ ), nitric oxide ( $\text{NO}\cdot$ ), and singlet oxygen ( $^1\text{O}_2$ )<sup>[16]</sup>. In general, most free radicals are derivatives of oxygen, such as superoxide.

During the normal aerobic metabolism, oxygen is the final electron acceptor at the end of the mitochondrial respiratory chain, during which it is fully reduced to water<sup>[17-19]</sup>. A small percentage of the electrons do not

travel to the end of the electron transport chain in the mitochondrial membrane (cytochrome oxidase); they escape and react directly with oxygen in the early part of the chain, forming  $\cdot\text{O}_2$ <sup>[20]</sup>. It has been estimated based on studies of isolated brain mitochondria that 1%-2% of the electrons that travel down the respiratory chain leak out and reduce oxygen to superoxide and its dismutation product, hydrogen peroxide ( $\text{H}_2\text{O}_2$ )<sup>[21,22]</sup>. Hydrogen peroxide is not a radical, but it serves as a precursor to the generation of the hydroxyl radical ( $\cdot\text{OH}$ ) in the presence of iron ions. Superoxide is also produced endogenously through the action of oxidase enzymes (*e.g.*, xanthine oxidase) in postischemic tissues<sup>[23,24]</sup>. Redox cycling substrates (*e.g.*, catecholamines), the cytochrome P450 system and soluble oxidases, such as nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase in phagocyte cells (*e.g.*, neutrophils and monocytes), are endogenous sources of superoxide production<sup>[17-19,25-27]</sup>. Arachidonic acid metabolizing enzymes (cyclooxygenase and lipoxygenase) are other sources of intracellular superoxide production<sup>[28-30]</sup>. Although superoxide serves as a precursor to many ROS (*e.g.*,  $\cdot\text{OH}$ ,  $\cdot\text{O}_2\text{H}$ ), it is not the most reactive of the species<sup>[15]</sup>. Figure 1 illustrates the chemical interconversions that are driven by  $\cdot\text{O}_2^-$ . Superoxide reacts with hydrogen to form hydrogen peroxide by nonenzymatic or by superoxide dismutase-catalyzed dismutation. One of the most important reactions for this is the  $\cdot\text{O}_2^-$  driven Fenton reaction, in which ferric or cupric ions ( $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ ), usually  $\text{Fe}^{3+}$ , are reduced by superoxide, and then, the reduced metal ion reacts with  $\text{H}_2\text{O}_2$  to form  $\cdot\text{OH}$ <sup>[7]</sup>. Another important free radical,  $\text{NO}\cdot$ , is synthesized from L-arginine by the enzyme nitric oxide synthase.  $\text{NO}\cdot$  can interact with  $\cdot\text{O}_2^-$  to generate another damaging molecule, peroxynitrite ( $\text{ONOO}\cdot$ ) (Figure 1). Once ROS are formed by the mechanisms explained in Figure 1, they can generate oxidative damage to a variety of biological molecules, such as lipids, proteins, and nucleic acids. The most important ROS involved in cellular oxidative damage is  $\cdot\text{OH}$ . Prime targets for ROS are the unsaturated fatty acids in membrane lipids, which will be discussed in the lipid peroxidation section. Proteins may also be damaged by ROS. This damage can be achieved through different stages, starting with site-specific lesions in the protein structure that leads to fragmentation products and cross-linked reaction products with other cellular components<sup>[25-27,31-33]</sup>. This oxidative damage to proteins by ROS can cause enzymatic dysfunction. For example, oxidation of the active site of the  $\alpha$ -protease inhibitor inactivates the protein itself, causing tissue damage<sup>[31]</sup>. Nucleic acids can also be affected by ROS. Oxidative damage to the DNA can occur after the attack of hydroxyl radicals on the sugar residues, yielding strand break products<sup>[31]</sup>. This may occur indirectly, when oxidative stress activates endonuclease, or directly, through the reaction of  $\text{H}_2\text{O}_2$  with DNA-bound metal ions and the formation of  $\cdot\text{OH}$ , which fragments the DNA at site-specific spots<sup>[15,31,34]</sup>.

ROS play an important role in increasing the intracellular levels of calcium. Ikebuchi *et al*<sup>[35]</sup> reported that the

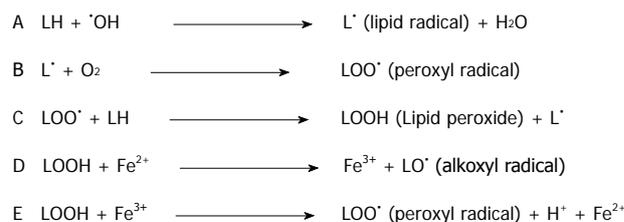


**Figure 1** Reactions of superoxide ( $\text{O}_2^-$ ) that generate highly reactive oxygen species.  $\cdot\text{O}_2^-$  can be dismutated into  $\text{H}_2\text{O}_2$ , which, in the presence of  $\text{Fe}^{2+}$ , can generate the highly reactive hydroxyl radical,  $\cdot\text{OH}$ .  $\cdot\text{O}_2^-$  may react with  $\text{NO}$  to form the strong pro-oxidant peroxynitrite ( $\text{ONOO} \cdot$ ); alternatively, it may react with  $\cdot\text{OH}$  to form singlet oxygen ( $\cdot^1\text{O}_2$ ); NADPH: Nicotinamide adenine dinucleotide phosphate.

superoxide anion activates calcium channels in human amnion cells to increase the intracellular calcium concentrations. Hirosumi *et al*<sup>[36]</sup> and others have shown that the superoxide anion also increases the intracellular calcium concentration in endothelial cells<sup>[37,38]</sup>. ROS inhibit the  $\text{Ca}^{2+}$ -ATPase, leading to elevated intracellular  $\text{Ca}^{2+}$  concentration<sup>[39]</sup>. Kimura *et al*<sup>[38]</sup> have found that  $\cdot\text{O}_2^-$  attenuated smooth muscle contraction by impairing  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  entry (CRAC), ATP-induced  $\text{Ca}^{2+}$  transient, and  $\text{Ca}^{2+}$  sensitivity in bovine aortic smooth muscle cells. Recent studies have shown that intracellular  $\text{Ca}^{2+}$  is involved in capsaicin-induced apoptosis. However, the molecular mechanisms of these intracellular  $\text{Ca}^{2+}$  signal pathways that lead to a sensitivity for the TNF-related induction of apoptosis remain unclear<sup>[40,41]</sup>. We will see in the next section that ROS are able to increase lipid peroxidation in the cell membrane to alter its permeability to ions and proteins.

## CONCEPTS OF OXIDIZED LIPIDS

Oxidized lipids, also known as lipid hydroperoxides and lipid peroxides, can be formed in the presence of oxygen free radicals. The double bonds of polyunsaturated fatty acids (PUFA), such as linoleic acid, are most susceptible to oxidation by ROS<sup>[42]</sup>. The peroxidation chain reactions of membrane PUFA begin when oxygen free radicals extract hydrogen atoms from the methylene carbons adjacent to carbon-carbon double bonds. The presence of the double bond in the fatty acids weakens the carbon-hydrogen bonds in the carbon atoms adjacent to the double bonds, thus allowing hydrogen to be removed in the presence of ROS<sup>[15]</sup>. Because a hydrogen atom has only one electron, the loss of hydrogen from a methylene group leaves behind an unpaired electron on the carbon



**Figure 2** Initiation and propagation of the free-radical chain reaction of lipid peroxidation. Reactions D and E represent the metal-catalyzed Fenton reaction.

atom adjacent to the double bond, and the lipid becomes a lipid radical ( $\text{L} \cdot$ ). Next, the reaction of lipid radicals with oxygen ( $\text{O}_2$ ) molecules forms a peroxyl radical ( $\text{LOO} \cdot$ ). Peroxyl radicals are capable of abstracting a hydrogen atom from adjacent lipids to form lipid hydroperoxides ( $\text{LOOH}$ ), sometimes called lipid peroxides. Once the chain of lipid peroxide formation is initiated, it becomes self-propagating. These events are illustrated in Figure 2. Reduced iron ( $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ) complexes can react with lipid peroxides to form strong oxidizing agents, such as alkoxyl radicals ( $\text{LO} \cdot$ ) and  $\text{LOO} \cdot$  (Figure 2D and E).

Lipid peroxides and oxygen radicals are responsible for many of the damaging reactions in the cell. They stimulate the peroxidation reactions that are toxic to cells and cell membranes. They can damage biological membranes, make the membrane leaky, and eventually cause complete membrane breakdown<sup>[43]</sup>. Furthermore, endothelial cell injury, the enhanced adhesion and activation of neutrophils, platelet aggregation, an increased uptake of low density lipoproteins by the endothelium, increased intracellular calcium and increased production of toxic aldehydes, as well as many other cellular dysfunctions, are due to the effects of  $\text{LOOH}$  and oxygen free radicals<sup>[42]</sup>. The breakdown products of lipid peroxidation, such as malondialdehyde, can affect membrane proteins by cross-linkage, rendering them useless as receptors or enzymes<sup>[44]</sup>. These different lipid peroxide effects can lead to cellular dysfunction and may result in the deaths of the affected cells.

## CONCEPTS OF ANTIOXIDANTS

An antioxidant is any substance or compound that scavenges oxygen free radicals or inhibits the oxidation process in the cell<sup>[45]</sup>. The level of antioxidant defenses available within the cells and extracellularly should be sufficient to oppose the toxic effects of lipid peroxides and to maintain normal physiology. This balance can be lost due to the over-production of free radicals or the inadequate intake of nutrients containing antioxidant molecules. Antioxidants can be considered plasma antioxidants and intracellular antioxidants.

The major role of plasma antioxidant defense is to bind transition metal ions, such as iron and copper, thereby lowering their plasma concentration and capacity to stimulate free radical reactions<sup>[15]</sup>. This prevents the formation of hydroxyl radicals of the very potent variety that is able

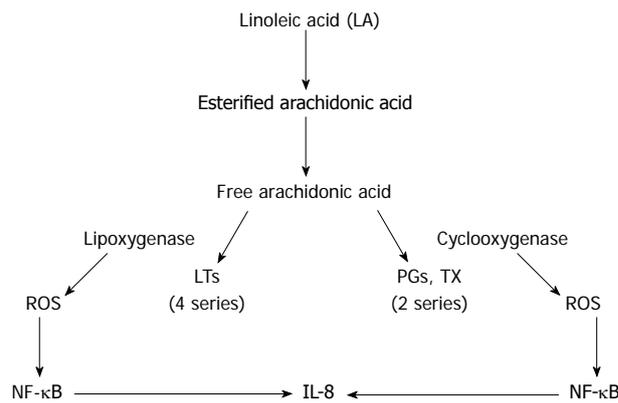
to generate lipid peroxides. This is achieved by plasma antioxidants, such as transferrin, lactoferrin, ceruloplasmin, albumin, uric acid, haptoglobins, and hemopexin<sup>[45]</sup>. Ascorbic acid is also a plasma antioxidant that can scavenge water-soluble peroxy radicals and other ROS<sup>[17-19]</sup>. This is accomplished through the completion of two reactions. First, the ascorbic acid is converted into the ascorbyl radical and then to dehydroascorbate. Ascorbic acid can also reduce  $\alpha$ -tocopheryl radicals to  $\alpha$ -tocopherol at the cell membrane surface<sup>[46]</sup>. Vitamin E ( $\alpha$ -tocopherol), a lipid peroxidation, chain-breaking antioxidant, is localized in membranes and lipoproteins<sup>[17]</sup>. It is the most important lipid-soluble antioxidant in the human plasma<sup>[47-50]</sup>. Vitamin E usually donates a hydrogen atom to the peroxy radical and prevents the propagation of the chain reaction of lipid peroxidation. Each tocopherol can react with two  $\text{LOO}^\bullet$ , the first  $\text{LOO}^\bullet$  reacts with  $\alpha$ -tocopherol to form the  $\alpha$ -tocopherol radical that reacts with the second  $\text{LOO}^\bullet$  to form a stable adduct ( $\text{LOO}-\alpha$ -tocopherol)<sup>[45]</sup>. Decreased blood and mucosal levels of vitamins A, C, E and  $\beta$ -carotene were reported in Crohn's patients<sup>[51-60]</sup>. Reimund *et al*<sup>[61]</sup> showed that antioxidants inhibited the production of inflammatory cytokines, such as IL-1 and  $\text{TNF}\alpha$ , in the colonic mucosa of inflammatory bowel disease patients. Vitamin C and E inhibited the levels of oxidative stress in human intestinal smooth muscle (HISM) cells isolated from Crohn's bowels<sup>[62]</sup>.

The main intracellular antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase. Human plasma has very little or no catalase, but it contains low activities of SOD and GSH-Px<sup>[15]</sup>. SOD is localized in both the cytosol (CuZn-SOD) and the mitochondria and acts to dismutate superoxides into hydrogen peroxide and molecular oxygen<sup>[17]</sup>. SOD is considered as the major intracellular enzyme because it is capable of reducing the most abundant free radical,  $\text{O}_2^\bullet$ . Catalase reacts rapidly with hydrogen peroxide, a precursor of  $\text{OH}^\bullet$  in the presence of iron, and converts it into water and molecular oxygen. Selenium glutathione peroxidase also converts hydrogen peroxide into water and lipid hydroperoxides into water and harmless fatty acid alcohols<sup>[45]</sup>. Decreased intestinal mucosal levels of CuZn superoxide dismutase and glutathione were reported in Crohn's patients<sup>[63-69]</sup>.

## OXIDANT STRESS IN CROHN'S DISEASE

Oxidant stress is a major factor in Crohn's disease. Reactive oxygen species can cause DNA modifications and damage, as briefly discussed in the previous section. The major sign of these modifications is the formation of 8-hydroxyguanine (8-OHDG). It was reported that 8-OHDG levels were significantly higher in the inflamed part of the bowel of Crohn's patient<sup>[70]</sup>. These data show an important indication of the role and existence of ROS in the pathogenesis of Crohn's disease.

There is direct and indirect evidence suggesting that the chronically inflamed intestines of Crohn's patients may be subjected to considerable oxidative stress.



**Figure 3** Two proposed mechanisms through which linoleic acid could increase the production of interleukin-8 via the activation of arachidonic acid pathways. By the generation of reactive oxygen species (ROS) from the cyclooxygenase and lipoxygenase enzymes to activate Nuclear factor- $\kappa$ B (NF- $\kappa$ B), increasing interleukin (IL)-8 production; or by the use of AA metabolites to increase IL-8 production. LTs: Leukotrienes; Tx: Thromboxane.

The direct evidence includes the demonstration of the increased production of ROS in response to various stimuli by phagocytic cells (neutrophils, monocytes, and macrophages) isolated from the inflamed bowels of patients with IBD (both Crohn's disease and ulcerative colitis)<sup>[7,71-73]</sup>. Kitahora *et al*<sup>[74]</sup> have demonstrated that monocytes from patients with Crohn's disease release large amounts of ROS during stimulation. It is well known that active IBD is characterized by persistent neutrophil infiltration into the injured site. The influx of neutrophils and macrophages into the bowel wall during inflammation causes a large degree of tissue damage due to their release of myeloperoxidase, which constitutes 5% of their total protein load. Myeloperoxidase metabolizes  $\text{H}_2\text{O}_2$  and chloride ions to form the potent oxidizing agent hypochlorous acid, commonly known as bleach, which is thought to be 100-1000 times more toxic than superoxide and hydrogen peroxide. Inflammatory phagocytes are activated by certain pro-inflammatory agents, such as leukotriene  $\text{B}_4$  ( $\text{LTB}_4$ ), platelet-activating factor (PAF), immune complexes, and bacterial products. As a result, the phagocytes release large amounts of ROS into the extracellular space<sup>[72-75]</sup>. Increased synthesis of  $\text{LTB}_4$  and PAF was detected in mucosal samples obtained from patients with active IBD<sup>[76-80]</sup>. Alzoghaibi *et al*<sup>[81]</sup> showed a significant increase in the production of the neutrophil-attracting chemokine, IL-8,  $\text{LTB}_4$  and other arachidonic acid metabolites in isolated HISM cells from Crohn's strictures after stimulation by linoleic acid or oxidized linoleic acid (OxLA). The cyclooxygenase and lipoxygenase enzymes generate ROS when activated. Both cyclooxygenase and lipoxygenase have been involved in the production of IL-8 from HISM cells (Figure 3). OxLA increased the levels of lipid peroxidation, an indicator of oxidative stress, as quantified by thiobarbituric acid reactive substances in HISM cells. These pro-inflammatory mediators activate certain receptors on the phagocytes' plasma membranes, resulting in the activation of plasma membrane-associated NADPH oxidase. NADPH oxi-

dase reduces oxygen molecules to superoxide on the cell membrane and releases the superoxide extracellularly.

Most of the evidence for the role of the ROS in Crohn's disease is indirect. Under normal physiological conditions, dietary and enzymatic antioxidants protect tissues from the damaging effects of ROS. However, a state of oxidative stress may exist when there is an imbalance in the levels of antioxidants and ROS. When the levels of antioxidants are low and ROS are high, increased cellular oxidative stress is present; such is the case in Crohn's disease<sup>[63,82-89]</sup>. The intestinal mucosa is particularly favored, as it is the location of many different enzymes necessary for the production of large amounts of ROS, such as xanthine oxidase, cyclooxygenase, and 5-lipoxygenase<sup>[75]</sup>. In addition to these sources of ROS, the number of the main sources of ROS (phagocyte cells) is increased in IBD<sup>[72,82]</sup>. For example, neutrophils account for up to 20% of the cells found in the lamina propria in active IBD<sup>[86]</sup>. On the other hand, Crohn's patients have low blood levels of vitamin C and vitamin E<sup>[63]</sup>. They also have low levels of CuZn superoxide dismutase, glutathione peroxidase, catalase, vitamin A and  $\beta$ -carotene in their intestinal mucosa, submucosa and muscularis-serosa<sup>[72,82-85,89]</sup>. This coincidence of increased levels of ROS and decreased levels of antioxidant defenses results in a state of oxidative stress in Crohn's strictures. A study of mucosal biopsies obtained from patients with IBD by Simmonds *et al*<sup>[86]</sup> demonstrated a significant increase in chemiluminescence, a very sensitive measurement for ROS, in comparison to controls. The addition of various antioxidant enzymes and scavengers, such as SOD, catalase, dimethyl sulfoxide (hydroxyl radical scavenger) and taurine (hydrochloride scavenger), confirmed that this increase in chemiluminescence was due to ROS. A study of HISM cells demonstrated a significant inhibition in TBARS after the cells had been exposed to antioxidant vitamins, such as C and E. Vitamin C and E also inhibited the effect of oxidized linoleic acid on the production of the most potent neutrophil-attracting chemokine, IL-8, from HISM cells<sup>[62]</sup>.

Clinical data suggest that ROS may play a role in the pathogenesis of Crohn's disease. Emerit and coworkers reported that intramuscular injections of bovine CuZn-SOD had a beneficial effect in attenuating mucosal inflammation and injury in Crohn's patients<sup>[90,91]</sup>. The beneficial effect of this antioxidant enzyme is a strong indication of the role of ROS in Crohn's disease. Another piece of evidence of the involvement of ROS in this disease is the use of 5-aminosalicylic acid (5-ASA) in treating patients with IBD. It is well known that the oral administration of sulfasalazine (SAZ) is capable of reducing the mucosal injury and inflammation associated with Crohn's disease. 5-ASA is the pharmacologically active moiety of SAZ<sup>[72,75]</sup>. It has been suggested that 5-ASA protects the gut mucosa by inhibiting cyclooxygenase and lipoxygenase activities. Several studies have reported that 5-ASA is a potent antioxidant and free radical scavenger. Research from several laboratories has demonstrated that 5-ASA is

very effective in scavenging hydroxyl radicals, interacting with superoxides and causing the rapid decomposition of this free radical<sup>[92,93]</sup>. Nielsen *et al*<sup>[71]</sup> demonstrated a high rate of lipid peroxidation in the gut mucosa obtained from patients with IBD. They also reported a significant inhibition in lipid peroxidation among patients who had been treated with SAZ for 5 wk<sup>[94]</sup>. These data implicate the involvement of ROS in Crohn's disease due to the antioxidant and free radical scavenging actions of 5-ASA or due to its role as an inhibitor of cyclooxygenase and lipoxygenase, which are enzymatic sources of ROS.

In summary, ROS could be an important factor in Crohn's disease. Crohn's disease is associated with an imbalance, comprising increased ROS and decreased net antioxidant activity. A deficiency in antioxidant molecules could lead to increased levels of lipid peroxides or ROS, which could act locally or be secreted into the circulation to produce different systemic effects in the patient. Obviously, more work is needed in this area to demonstrate the effects of ROS on the production of other neutrophil chemokines from different layers of Crohn's strictures. Future research should also address the question of whether ROS are involved in increasing the production of the different extracellular proteins, such as collagen, that lead to increased thickness of the bowel wall and decreased bowel diameters by enhancing the transcriptions of certain genes using specific transcription factors.

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## Current position of ALPPS in the surgical landscape of CRLM treatment proposals

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### Abstract

The Authors summarize problems, criticisms but also advantages and indications regarding the recent surgical proposal of associating liver partition and portal vein ligation (PVL) for staged hepatectomy (ALPPS) for the surgical management of colorectal liver metastases. Looking at published data, the technique, when compared with other traditional and well established methods such as PVL/portal vein embolisation (PVE), seems to give real advantages in terms of volumetric gain of future liver remnant. However, major concerns are raised in the literature and some questions remain unanswered, preliminary experiences seem to be promising. The method has been adopted all over the world over the last 2 years, even if oncological long-term results remain unknown, and benefit for patients is questionable. No prospective studies comparing traditional methods (PVE, PVL or classical 2 staged hepatectomy)

with ALPPS are available to date. Technical reinterpretations of the original method were also proposed in order to enhance feasibility and increase safety of the technique. More data about morbidity and mortality are also expected. The real role of ALPPS is, to date, still to be established. Large clinical studies, even if, for ethical reasons, in well selected cohorts of patients, are expected to better define the indications for this new surgical strategy.

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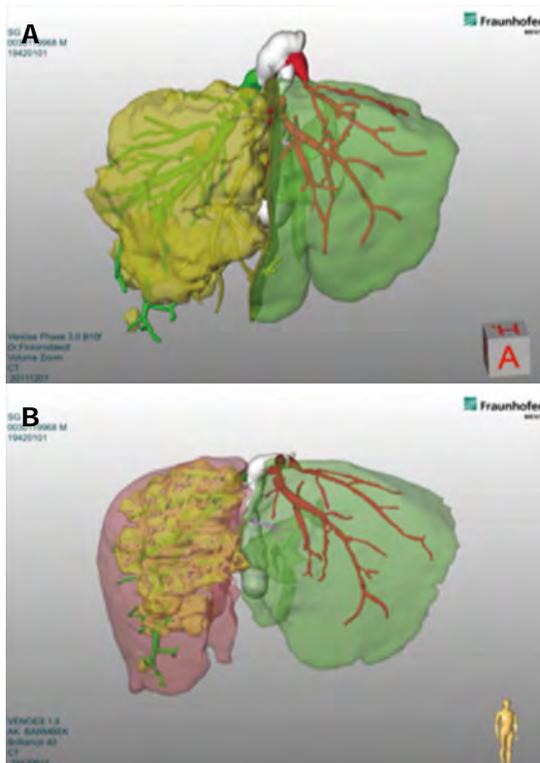
**Key words:** Portal ligation; *In situ* split; Liver resections; Colorectal metastases; Liver metastases

**Core tip:** The recent publication by Regensburg's Group on the new technical possibility of associating liver partition and portal vein ligation for staged hepatectomy for the surgical managing of bilateral colorectal liver metastases, generated a great debate and a burst of publications about preliminary experiences from many groups all over the world. As one of the first groups in Germany to adopt this technique, in the present article we clarify some aspects of our experience in the light of published data and raised concerns.

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### INTRODUCTION

The progressive enlargement of surgical indications to colo-rectal liver metastasis (CRLM) resection of the last 20 years has led to a redefinition of resectability criteria<sup>[1]</sup>. Nowadays no limits due to number of lesions and



**Figure 1** Example of strategy using a semi-automated 3D volumetry system. Hepavision® MEVIS. A: 3D volumetry before associating liver partition and portal vein ligation for staged hepatectomy (ALPPS): future liver remnant (FLR) 26%; B: Two weeks after ALPPS volumetry of FLR: 43% (increase of 65%).

location are of value as in the past<sup>[2]</sup>. The main problem for resectability criteria of CRLM is due to volume of future liver remnant<sup>[3]</sup>, in fact, postoperative liver failure is one of the biggest risk and a significant complication after extended hepatectomy<sup>[4]</sup>. Some techniques were established such as portal vein ligation (PVL) and portal vein embolisation (PVE) with well known advantages and limits. Also 2 stage combined strategies were developed to overcome resectability problems due to bilobar location of metastases and two-staged hepatectomy has been reported as an efficient strategy for oncological outcomes and has been adopted by many liver centers<sup>[5,6]</sup>. A real novelty on 2 staged surgical procedures that has recently been proposed, is the advent of associating liver partition and PVL for staged hepatectomy (ALPPS)<sup>[7]</sup>. The technique was first performed by Schlitt *et al.*<sup>[8]</sup> of Regensburg in 2007 and first presented to a German Congress in 2010. After that the technique spread all over the world<sup>[9]</sup>. This is rapidly gaining great interest from the surgical community leading to debate<sup>[10]</sup> and even proposals for a reinterpretation of methods<sup>[10-12]</sup>, and giving a new hope to a large number of patients traditionally judged unresectable<sup>[13]</sup>. Despite an “explosion” of publications and case reports in the literature during the last 2 years, a lot of questions remain concerning safety and effectiveness of this method<sup>[14]</sup>. Given the large amount of surgical experience world wide<sup>[15]</sup>, and the critical appraisals of some surgical groups, in the present article we would like to summarize the main sur-

gical aspects, open questions and express our point of view on this method in the light of our preliminary experience<sup>[16]</sup> and other published data, in the era of two-stage treatment of CRLM<sup>[17]</sup>.

## TECHNICAL ASPECTS AND DEVELOPMENT

The first aim of introducing this technique by Regensburg's group was to enhance liver hypertrophy after portal ligation increasing the ischemia effect on future liver remnant (FLR)<sup>[16]</sup>. The concept on which this technique is based seems to be an old finding<sup>[18]</sup>, but of course revisited and reinvented or recombined in the light of new problems of CRLM surgery<sup>[19]</sup>. The assumption is that any closure of the right portal branch is followed by a reactive perfusion of “deportalized” liver, from contralateral one, through the intrahepatic branches and collaterals presents between the 2 lobes<sup>[20]</sup>. This aspect was recently confirmed by a clinical study<sup>[21]</sup>.

The technique consists in an association of classical right portal branch ligation together with liver parenchyma surgical split. The split could be conducted following the falciform ligament line (splitting segment 2-3 from the rest of the liver) as originally described<sup>[7]</sup>, or even atypically adding 4b segment as previously shown in our video<sup>[22]</sup>. To split the liver avoiding manipulation and obtaining a better bleeding control, such as a clear anatomic line of parenchymal transection, we usually perform an anterior approach<sup>[23]</sup>. Between the right and left split hemilivers a plastic sheet or bag should be positioned in order to avoid cicatrization with the disappearance of the resection line. In addition some atypical resection of additional metastases (1 or 2) in the future liver remnant must be performed. We have extended the indication to ALPPS also for bilateral CRLM with little FLR (< 30%) or even < 40% with damaged liver parenchyma; and during the first step of the procedure we resect metastases on FLR. Other groups used more restricted indications reserving ALPPS only for patients without metastases on FLR<sup>[7]</sup>.

In the original description the Authors waited for about 8-10 d before performing the 2<sup>nd</sup> step of specimen removal<sup>[7]</sup>. Other Authors usually wait 7 d<sup>[24]</sup>, while our group usually performs the 2<sup>nd</sup> step after 12 d. A computed tomography (CT) scan after 1<sup>st</sup> step, in 7-10 d, is mandatory in order to evaluate volumetric gain. We routinely use 3D-reconstruction and Volumetry performed by MEVIS® system using Hepavision® software<sup>[22]</sup> (Figure 1). The key role of CT-volumetry with 3D-reconstructions for this novel method was later confirmed by another study<sup>[25]</sup>. This technique showed that the speed of hypertrophy and also the percentage of volume gain were more enhanced than with classical methods such as PVE or PVL<sup>[7]</sup>.

Some technical variants were quickly introduced by some surgeons. Some authors, in fact, proposed the laparoscopic approach<sup>[11,26]</sup>, citing some advantages of lapa-

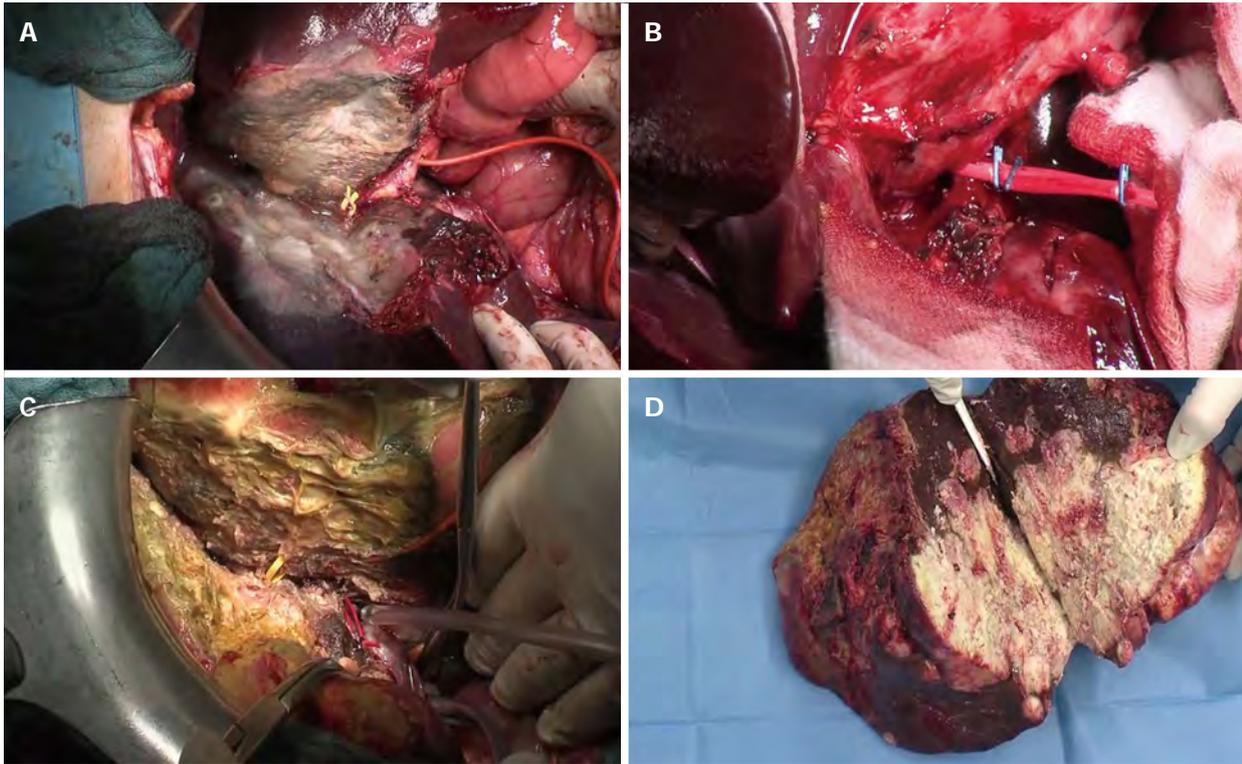


Figure 2 Intraoperative images of associating liver partition and portal vein ligation for staged hepatectomy in our center. A, B: 1<sup>st</sup> Step procedure with apposition of colored loop (red for the right hepatic artery, yellow for the right hepatic duct) and of T-drainage. C, D: Easy identification of loops during the second step and opened specimen of R0-resection of colo-rectal liver metastasis.

roscopy in avoiding adhesions thus making the 2<sup>nd</sup> step easier<sup>[27]</sup>. Even if ALPPS was developed for extended right hepatectomy, 3 main strategies were recently standardized to use ALPPS not only for right hepatectomies but even for extended left hepatectomies<sup>[28]</sup>.

We proposed the following technical modifications in addition to referred routine use of 3D-reconstruction in order to improve the safety of the procedure: (1) Do not use the plastic bag or sheet but interpose only a fibrillar mesh (Figure 2A); (2) Use of a colored plastic loop to leave *in situ* for the 2<sup>nd</sup> step (yellow for right biliary duct and red for hepatic artery) in order to find it very easily and quickly during the challenging 2<sup>nd</sup> step (Figure 2B and C); and (3) Routine use of T-drain in order to reduce the risk of biliary leak and biomass (main referred surgical complication after preliminary reports (Figure 2B and C). Additionally we enhanced the significance of complete liver mobilization (apparently in contradiction with the anterior approach!) as previously described as a preliminary maneuver of surgical step 1, that in this technique, in our opinion, is of value not only for a complete manual and ultrasonography exploration of both lobes, but also to enhance the ischemia effect, avoid collateralisation through ligaments' vessels and increase the hypertrophy effect of ALPPS. On the other hand some surgeons criticized<sup>[10,15]</sup> the technique, questioning: complexity of procedure, high risk of 2 very close big operations, additional morbidity and reported mortality, and uncertainty of long-term oncological results. If detractors have made

some logical considerations about the proposed method, also enthusiasts of ALPPS have been engaged in reporting positive results<sup>[11,16,17]</sup>. It should be underlined that some of the most talented surgeons use ALPPS<sup>[9]</sup>, testifying to the potential of this technique, trying to find the best indications (Table 1).

We have also expressed some considerations related to our preliminary data in a previous article<sup>[16]</sup>, however, the ideas and criticisms raised during this last year convinced us to introduce, in this debate, some other considerations.

## PUBLISHED EXPERIENCE

First of all it should be taken into consideration that ALPPS is mainly indicated for patients that have to undergo a right trisegmentectomy. This extended liver resection is known to be at particular risk of postoperative liver failure<sup>[4]</sup>, the combination in a 2 staged procedure is forced in these patients by a judgment of not resectability with other established 2 stage surgical strategies<sup>[29-31]</sup>. Therefore this cohort of patients is "*per se*" a group of very sick patients with advanced disease, traditionally not resectable CRLM and therefore destined for palliative treatment. The novelty of this method is the percentage gain of patients to resectability. Thus we are convinced that the questionable additional risk related to the technique could be acceptable in the light of resectability gain. Doubtless, also other one stage combined proposed

**Table 1** Associating liver partition and portal vein ligation for staged hepatectomy: an overview of worldwide experience

Author	Cases (n)	Hypertrophy (range)	Days of interval (range)	Morbidity	Mortality
Schnitzbauer <i>et al</i> <sup>[7]</sup>	25	74% (21-192)	8 (4-8)	64%	12%
Conrad <i>et al</i> <sup>[11]</sup>	1	44.80%	9	0%	0%
Robles Campos <i>et al</i> <sup>[12]</sup>	1	57%	7	0%	0%
Dokmak <i>et al</i> <sup>[15]</sup>	8	70% (5-147)	7	87.50%	25%
Donati <i>et al</i> <sup>[16]</sup>	8	80% (66-200)	10 (7-21)	-	-
Knoefel <i>et al</i> <sup>[17]</sup>	7	63%	6 (4-8)	57.20%	14.20%
Machado <i>et al</i> <sup>[26]</sup>	8	88% <sup>1</sup>	9	0%	0%
Machado <i>et al</i> <sup>[27]</sup>	1	159%	21	0%	0%
Hahn <i>et al</i> <sup>[37]</sup>	1	94%	9	0	0
Sala <i>et al</i> <sup>[49]</sup>	10	82% (31-140)	7	40%	0%
Andriani <sup>[50]</sup>	2	-	30	0%	0%
Torres <i>et al</i> <sup>[51]</sup>	1	-	-	-	-
Li <i>et al</i> <sup>[52]</sup>	9	87.20%	13	22.20%	22.20%

<sup>1</sup>Data referred only to one case.

strategies could be taken into account in selected groups of patients<sup>[32]</sup>. However, very aggressive chemotherapeutic regimens, in many cases, are nowadays forcing surgeons to find new technical solutions, sometimes delaying radical treatment in order to achieve patients' safety. We also think that the potential of ALPPS was wrongly judged by some eminent colleagues only because it was tested on very challenging indications (duodenocephalopan-creatotomy and extended hemihepatectomy for biliary tract tumors)<sup>[15]</sup> leading to high morbidity and mortality rates. We expressed our opinion that the main indication for ALPPS seems to be for CRLM in selected patients, as stated in other papers<sup>[16]</sup>. In patients affected by bilateral CRLM, the proposed method regained resectability also in apparently not resectable patients, increasing safety of resection and lowering risk of postoperative liver failure, enhancing, in a very short time (average about 7 d), the great hypertrophy potential of liver parenchyma. Undoubtedly ALPPS, compared to traditional PVE or PVL, allows a tremendously quick FLR growth (22% *vs* 3% growth the day after the procedure)<sup>[16]</sup>. Of course our previous considerations should be considered in the light of no published long-term oncologic results. In consideration of the right moment to resect after split, this aspect should be carefully taken into account in patients submitted to several cycles of neoadjuvant chemotherapy. As we demonstrated in a previous publication, a long wait (about 4 wk) after split can allow a volumetric gain of 200%, but with the disadvantage of a more difficult second surgical step<sup>[23]</sup>. It is foreseeable that with the rapid diffusion of new neoadjuvant chemotherapeutic regimens (chemo first approach), the need for ALPPS, as a safe alternative strategy to the classic 2 stage approach, will increase; in fact, more patients are gaining, and will gain, resectability due to partial or sometimes full response to new chemotherapeutic protocols<sup>[33,34]</sup>. Thus a multidisciplinary approach of CRLM<sup>[35]</sup>, starting with aggressive neoadjuvant regimen, under indications of institutional tumor boards, will push more patients to ALPPS. On the other hand, ALPPS makes the resection safer after neoadjuvant chemothera-

py, reducing the risk of postoperative liver failure.

## OPEN QUESTIONS

Even with the big enthusiasm for this technique and surge in the number of centers adopting it over the last 2 years, even in an episodic manner, leading to a lot of case reports<sup>[36-38]</sup>, some considerations must be made. First of all we should consider that the method has not yet been tested in an evidence based manner, only preliminary experience is available, publishing dishomogeneous data. Even the technique has not yet been standardized. The big question is if oncologic long-term results are acceptable, if a gain, in terms of quality of life and time gained, could balance the big risk of complications and mortality. Whether the stimulation of liver hypertrophy could also accelerate tumor progression is also an open question still debated since the time of classic PVE, PVL techniques<sup>[39]</sup>. Recently, Van Gulik's group has shown how tumor progression could clearly be stimulated by PVE only<sup>[40]</sup>, reporting the objections about the short time-frame of ALPPS among speculations, because the same phenomenon is observed in PVE and PVL<sup>[41]</sup>. Even a study by Pamecha *et al*<sup>[42]</sup> first experimentally and then confirmed by Maggiori *et al*<sup>[43]</sup>, showed a clear tumor progression after PVE so that about one third of patients cannot undergo the second step after embolization because of tumor progression. Obviously one could speculate that if ALPPS allows a bigger and quicker liver regeneration, the same stimulation could realize an intensive and quicker tumor progression. An early recurrence was occasionally reported among disomogeneous published experiences<sup>[7]</sup>, but more data are expected in the next years and of course also results of international register.

If CRLM is to be considered the best indication in other tumours (for example Neuroendocrine tumor metastases) with slower biology and tumor progression, patients could benefit from such a method, this remains one of the main questions. Thus the great advantage of volumetric gain must be taken with the above mentioned

open questions before establishing the method in clinical practice. The method remains very challenging and not only for liver surgeons, but for extremely skilled liver centers and must be approached in a multidisciplinary manner. More effort must be made to reduce the morbidity and mortality associated with ALPPS.

Of course it must be taken into consideration that some other established methods of obtaining FLR hypertrophy are less risky and whenever possible should be the first choice in planning staged surgical strategies<sup>[44]</sup>. Nevertheless, also if PVE is, to date, the most used technique and considered the standard procedure to enhance FLR, about 30% of patients never undergo complete resection because of insufficient hypertrophy or tumor progression on FLR. Furthermore, as in a recent systematic review the two stage hepatectomy with traditional strategies has shown an certain morbidity, that appears comparable with the morbidity of ALPPS (17% after first step and 40% after the second one)<sup>[6]</sup>. Therefore, in well selected cases, in limit-cases, or when PVE failed to gain volumetric enhancement of FLR<sup>[17,45]</sup>, sometimes ALPPS seems to be the only reasonable-feasible option to achieve resectability. The additional morbidity and mortality referred in the bigger reports respectively up to 44% and 12% could be accepted as additional risks only in the light of “no other choices”, even if we need more scientific studies to confirm this. Furthermore, it should be taken into account that reported high mortality rates are referred to very initial experiences in very small groups of patients, in which also 1 death strongly influences overall mortality rates. However, also 3<sup>rd</sup> referral hepatobiliary centers need a learning curve to optimize the procedure. Some proposed technical details could reduce the “surgical risk” also shortening the time of the second procedure<sup>[15]</sup>. Due to ethical limits to clinical experimentation and the difficulty in recruiting a reasonable group of highly selected patients, an online world register was created (see international register: [www.alpps.net](http://www.alpps.net)). Some detractors of ALPPS have recently published a study comparing traditional PVE efficacy and safety compared with published data on ALPPS, concluding to be in favour of traditional and well established strategies<sup>[46]</sup>. However, despite confirming that PVE and similar techniques are still the standard of care, the referred study suffers from some BIAS in comparison, therefore conclusions are not well addressed by the study and a definitive conclusion cannot be made. The challenge of the ongoing study will be, despite the BIAS of patient collection from many different centers, to establish some kind of evidence of safety (as declared by many authors and criticized by others), usefulness (long-term oncologic results), best indications, and in our opinion also guidelines to standardize the preoperative flow-chart, surgical timing and steps. It is foreseeable that ALPPS could gain a position also among feasible surgical strategies for the complex scenario of Klatskin tumours<sup>[47,48]</sup> in order to extend the resectability rate as we stated in a previous article<sup>[16]</sup>, and as confirmed by recently reported experiences<sup>[35,49]</sup>. Despite all the potential of this technique, to

date the scientific evidence should be still considered as a phase 1 clinical trial; we believe that the method, in consideration of all the above mentioned open questions at the moment, should be adopted only by extremely well-trained and experienced Hepatobiliary Surgical Centres.

## CONCLUSION

The problems on the table are many and the technique needs to be defined, maybe first on acceptable indications and long-term results, in order to achieve the current position of ALPPS not only in the surgical management of CRLM, but also in its greater potential to treat other liver tumours. Therefore, in conclusion, we think that the ALPPS proposal should be considered the “real novelty” in the CRLM surgical landscape of the last 3 years and despite the enthusiastic view to “change the face of liver surgery” as suggested by other Authors<sup>[10]</sup>, we prefer to say that it is foreseeable that such a method will gain, after the physiological period of experimentation and publication of the first large clinical studies, an important position among the surgical strategy options for the surgeon managing bilateral colorectal liver metastases, even maybe for a restricted and well-selected subgroup of patients.

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## Pleiotrophin promotes perineural invasion in pancreatic cancer

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### Abstract

Perineural invasion (PNI) in pancreatic cancer is an important cause of local recurrence, but little is known about its mechanism. Pleiotrophin (PTN) is an important neurotrophic factor. It is of interest that our recent experimental data showed its involvement in PNI of pancreatic cancer. PTN strongly presents in the cytoplasm of pancreatic cancer cells, and high expression of PTN and its receptor may contribute to the high PNI of pancreatic cancer. Correspondingly, PNI is prone to happen in PTN-positive tumors. We thus hypothesize that, as a neurite growth-promoting factor, PTN may promote PNI in pancreatic cancer. PTN is released at the time of tumor cell necrosis, and binds with its high-affinity receptor, N-syndecan on pancreatic nerves, to promote neural growth in pancreatic cancer. Furthermore, neural destruction leads to a distorted neural homeostasis. Neurons and Schwann cells produce more N-syndecan in an effort to repair the pancreatic nerves.

However, the abundance of N-syndecan attracts further PTN-positive cancer cells to the site of injury, creating a vicious cycle. Ultimately, increased PTN and N-syndecan levels, due to the continuous nerve injury, may promote cancer invasion and propagation along the neural structures. Therefore, it is meaningful to discuss the relationship between PTN/N-syndecan signaling and PNI in pancreatic cancer, which may lead to a better understanding of the mechanism of PNI in pancreatic cancer.

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**Key words:** Pleiotrophin; N-syndecan; Neurite outgrowth; Perineural invasion; Pancreatic cancer

**Core tip:** We discussed the important novel role of pleiotrophin (PTN) in perineural invasion (PNI) of pancreatic cancer, an important cause of local recurrence. Our recent experimental data demonstrated the involvement of PTN in PNI of pancreatic cancer. PTN strongly presents in the cytoplasm of pancreatic cancer cells, and high expression of PTN and its receptor may contribute to the high PNI of pancreatic cancer.

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### INTRODUCTION

Pancreatic cancer is one of the most malicious human malignancies with the lowest 5-year survival rate<sup>[1-3]</sup>. At the time of diagnosis, most patients have locally advanced disease and/or distant metastatic lesions precluding radical operation resection<sup>[3-5]</sup>. Perineural invasion (PNI) is considered as an important factor of aggressive tumor

behavior, and it is associated with local recurrence and poor outcome of pancreatic cancer<sup>[6]</sup>. Pancreatic cancer cells frequently have intimate contact with intrapancreatic nerves and thereby alter, invade, and damage the intrapancreatic nerves<sup>[7]</sup>. PNI extending into the extrapancreatic nerve plexus is a histopathologic characteristic in pancreatic cancer, which leads to abdominal pain and retropancreatic tumor extension<sup>[8-10]</sup>. PNI is defined as presence of cancer cells within the epineural, perineural, and endoneurial spaces of the neuronal sheet and around the nerves<sup>[11,12]</sup>. It precludes curative resection, promotes local recurrence, and finally negatively influences the prognosis of the patients. However, the mechanisms of the alteration and invasion of pancreatic nerves and the spread of cancer cells along pancreatic nerves in pancreatic cancer remain poorly understood. Therefore, neurotrophic factors are of interest because recent experimental data showed their involvement in neuro-cancer interactions in pancreatic cancer<sup>[13]</sup>.

## MECHANISM OF PNI IN PANCREATIC CANCER

The mechanism of PNI in pancreatic cancer is unclear, although it can be partially explained by the anatomical proximity of the pancreatic and celiac artery neural plexus. The perineurium is believed to be deficient near the nerve ending, at the site invaded by the blood vessels in the nerves, and at the site invaded by reticular fiber<sup>[14]</sup>. Another possible explanation of PNI in pancreatic cancer is neurotropism because some advanced cancers with PNI express numerous types of neuroendocrine markers including S-100, Synaptophysin, substance P, enkephalin, and neural cell adhesion molecules<sup>[15]</sup>. Other specific factors such as nerve growth factor also enhance the cancer-nerve interaction, providing biological and physical parameters that would explain their frequent and intimate relationship<sup>[16]</sup>.

## PLEIOTROPHIN-CANCER INTERACTION

Pleiotrophin (PTN) is a neurotrophic factor, also known as the neurite growth-promoting factor. The protein is mainly expressed during early embryogenesis. In human adult tissues, it is markedly down-regulated and present only at minimal levels in very few tissues. PTN is a 136-amino-acid long secreted cytokine related to diverse biological properties, including neurite outgrowth, angiogenesis, and tumor growth<sup>[17,18]</sup>. It is strongly expressed in different human tumor cells, and expression of the PTN gene in tumor cells *in vivo* accelerates growth and stimulates tumor angiogenesis<sup>[19,20]</sup>. Experimental evidence from different laboratories also supported the potential of PTN to play an important role in promotion of human tumors. PTN transcripts are highly expressed in a high proportion of different human tumor samples, including pancreatic cancer, breast carcinoma, melanocytic tumor, carcinoma of the prostate, glioblastoma, and astrocytomas<sup>[21-25]</sup>. Cell

lines derived from these tumors have constitutive activation of the endogenous *PTN* gene, while PTN expression is not detected in non-tumor cell lines of the same origin and in the non-tumorous tissues<sup>[26]</sup>.

## ASSOCIATION BETWEEN PTN AND PANCREATIC CANCER

PTN is not expressed in normal pancreatic tissues, but it is highly expressed in pancreatic cancer tissues and correlates with pancreatic cancer progression<sup>[27]</sup>. In previous experiment, we studied PTN and its receptor N-syndecan protein levels in 38 patients with pancreatic cancer by immunohistochemistry, analyzed for its correlation with clinicopathological features, PNI, and prognosis. The results suggested that PTN was strongly present in the cytoplasm of pancreatic cancer cells; N-syndecan was intensely present in the perineurium of pancreatic nerves but not in the cancer cells. PTN combined with N-syndecan might have contributed to the high level of PNI and poor prognosis of pancreatic cancer<sup>[28]</sup>. Furthermore, tissue expression of PTN resulted in its elevated serum levels in more than 50% of the pancreatic cancer patients, and a statistically significant positive association was found between elevated serum levels of PTN at the time of surgery and its expression by tumors<sup>[27]</sup>. In both mice and humans, serum PTN levels dropped after successful tumor removal, suggesting that PTN may represent a new tumor marker in pancreatic malignancies.

## PTN-NERVE INTERACTION

PTN was initially isolated from neonatal rat brain as a neurite outgrowth-promoting protein. Previous studies have demonstrated that N-syndecan acts as a receptor in PTN-induced neurite outgrowth in perinatal rat brain neurons<sup>[29]</sup>. N-syndecan-stably-transfected N18 neuroblastoma cells showed clearly enhanced neurite outgrowth upon contact with PTN-containing substrate. PTN and N-syndecan utilize the cortactin-src pathway for the intracellular signaling in neurite outgrowth<sup>[30]</sup>.

PTN promoted neurite outgrowth from different cultured neuronal cell types, including cultures of embryonic and perinatal cortical neurons, neuroblastoma cells, and PC12 cells<sup>[31]</sup>, and anti-PTN antibodies inhibited neurite outgrowth *in vitro*<sup>[29]</sup>. The addition of PTN to donor cells resulted in better functional recovery and better survival of dopaminergic neurons, owing to the decrease of cell death after transplantation<sup>[32]</sup>. The results revealed that PTN had effects on donor cells in neural transplantation both *in vitro* and *in vivo*. In adult animals, PTN expression was lower but increased during recovery from injury, playing a major role in the cell growth and differentiation associated with tissue regeneration. A higher PTN level was noticed in sciatic nerves within a few days after crush injury when axon regrowth was induced, whereas PTN level was lowered after the axons reached their target<sup>[33]</sup>. The increased PTN protein levels during the first step of

peripheral nerve regeneration suggested time-restricted synthesis of PTN within the injured nerve. These results suggested that PTN may be involved in peripheral nerve regeneration after the nerve injury.

PTN and N-syndecan act as a ligand-receptor pair in neurite outgrowth<sup>[34]</sup>. It is possible that PTN and its receptor act synergistically to promote PNI in pancreatic cancer. Our previous experiments also showed that recombinant adenovirus-mediated PTN-shRNA successfully silenced *PTN* gene expression in pancreatic cancer cells, and the neurite outgrowth of dorsal root ganglion neurons was evidently inhibited by knocking down the PTN protein<sup>[35,36]</sup>.

## CONCLUSION

Previous studies described the importance of individual neurotrophic factor in PNI in pancreatic cancer<sup>[37]</sup>; however, the mechanism of PNI was not clarified explicitly. Former studies of PTN focused on angiogenesis, neuritis outgrowth, and tumor growth<sup>[38,39]</sup>. There was no relevant report about the association between PTN and PNI in human tumors. Interestingly, elevated PTN expression has been found to be an essential autocrine and paracrine factor for various human malignancies, including pancreatic cancer, breast carcinoma, melanocytic tumor, carcinoma of the prostate, and astrocytomas<sup>[40]</sup>. Correspondingly, PNI is also prone to happen in these PTN-positive tumors. Therefore, we hypothesize that, as a neurite growth-promoting factor, PTN and N-syndecan act synergistically to promote PNI in pancreatic cancer. PTN is an important factor of the induction of neurite outgrowth, survival of neurons, and peripheral nerve regeneration under pathological conditions<sup>[30,31,41]</sup>. PTN is released at the time of tumor cell necrosis and binds with its high-affinity receptor, N-syndecan on pancreatic nerve, to promote neurite growth in pancreatic cancer. Furthermore, in pancreatic cancer, cancer cells infiltrate and destroy the perineurium of pancreatic nerves, and the neural destruction leads to a distorted neural homeostasis. Neurons and Schwann cells produce more N-syndecan in an effort to repair the pancreatic nerves. However, the abundance of N-syndecan further attracts PTN-positive cancer cells to the site of injury, creating a vicious cycle. Ultimately, increased PTN and N-syndecan levels, due to the continuous nerve injury, may promote cancer invasion and propagation along the neural structures.

## FUTURE IMPLICATION

Pancreatic cancer is characterized by PNI, early lymph node metastasis, and poor prognosis. PNI is an important cause of local recurrence, but little is known about its mechanism. It is meaningful to discuss the relationship between PTN/N-syndecan signaling and PNI in pancreatic cancer, which will probably lead to a better understanding of the mechanism on PNI. Considering

that the production of inhibitors for PTN and N-syndecan is at the stage of laboratory trials, we believe that such study has significant translational potential. Due to the unclear mechanism, it is difficult to improve or apply gene therapy targeting the possible candidate cancer genes. Therefore, understanding the relationship between PTN/N-syndecan signaling and PNI may contribute to an improved therapy of PNI in pancreatic cancer. In further studies, we will silence the *Ptn* gene in orthotopic pancreatic cancer model in nude mice using recombinant adenovirus-mediated PTN-shRNA, and investigate the effects of PTN on PNI of pancreatic cancer.

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## Effects of integrin-targeted photodynamic therapy on pancreatic carcinoma cell

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### Abstract

**AIM:** To investigate the effects of photodynamic therapy with quantum dots-arginine-glycine-aspartic acid (RGD) probe as photosensitizer on the proliferation and apoptosis of pancreatic carcinoma cells.

**METHODS:** Construction of quantum dots-RGD probe as photosensitizer for integrin-targeted photodynamic therapy was accomplished. After cells were treated with photodynamic therapy (PDT), the proliferation of SW1990 cells were measured by methyl thiazolyl tetrazolium assay. Morphologic changes, cell cycle retardance and apoptosis were observed under fluoro-

scope and flow cytometry. The expression of myeloid cell leukemia-1 (Mcl-1), protein kinase B (Akt) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mRNA were detected by reverse transcription-polymerase chain reaction. The amount of reactive oxygen species were also evaluated by fluorescence probe.

**RESULTS:** The photodynamic therapy with quantum dots-RGD probe as photosensitizer significantly inhibited cell proliferation ( $P < 0.01$ ). Apoptotic cells and morphologic changes could be found under optical microscope. The FCM revealed PDT group had more significant cell apoptosis rate compared to control cells ( $F = 130.617, P < 0.01$ ) and cell cycle  $G_0/G_1$  and S retardance ( $P < 0.05$ ) compared to control cells. The expression of Mcl-1 and Akt mRNA were down-regulated, while expression of TRAIL mRNA was up-regulated after cells treated with PDT. PDT group had more significant number of cells producing reactive oxygen species compared to control cells ( $F = 3262.559, P < 0.01$ ).

**CONCLUSION:** The photodynamic therapy with quantum dots-RGD probe as photosensitizer significantly inhibits cell proliferation and increases apoptosis in SW1990 cells.

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**Key words:** Pancreatic carcinoma; Targeted probe; Photodynamic therapy; Apoptosis; Reactive oxygen species

**Core tip:** Arginine-glycine-aspartic acid (RGD), sequence of small peptide, is an integrin antagonist. Quantum dots are characterized by conjugation with antibodies, peptides, or small molecules. Therefore, the construction of RGD-coupled quantum dots fluorescence probe allows for successful combination to integrin. Photodynamic therapy with a quantum dots-RGD probe, as a photosensitizer, significantly inhibits cell proliferation and increases apoptosis in SW1990 cells.

Zhou M, Ni QW, Yang SY, Qu CY, Zhao PC, Zhang JC, Xu LM. Effects of integrin-targeted photodynamic therapy on pancreatic carcinoma cell. *World J Gastroenterol* 2013; 19(39): 6559-6567 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6559.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6559>

## INTRODUCTION

Pancreatic adenocarcinoma ranks as the fourth most common cause of cancer death in the United States. Patients usually present late with advanced disease, limiting attempted curative surgery to 10% of cases. Overall prognosis is poor with one-year survival rates of less than 10% with palliative chemotherapy and/or radiotherapy<sup>[1-3]</sup>. Given these dismal results, a minimally invasive treatment capable of local destruction of tumor tissue with low morbidity may have a place in the treatment of this disease. The photodynamic therapy is a treatment that has high specificity, minimal invasiveness and good cosmetic outcome, which produces local necrosis of tissue with light after prior administration of a photosensitizing agent<sup>[4]</sup>. In the past few years, little studies have been reported in the literature about the photodynamic therapy (PDT) on pancreatic cancer treatment, especially for the targeted-PDT<sup>[5-7]</sup>. Integrins  $\alpha_v\beta_3$  plays a critical role in regulating tumor growth and metastasis as well as tumor angiogenesis<sup>[8,9]</sup>. Arginine-glycine-aspartic acid (RGD) peptides is the integrin antagonist which can link to integrin<sup>[10-12]</sup>. Therefore, it makes the RGD coupled quantum dots (QDs) probe successful to image the pancreatic cancer. In order to further discuss the possibility of PDT applied to pancreatic cancer treatment and its mechanism, we investigated the effects of photodynamic therapy with quantum dots-RGD probe as photosensitizer on the proliferation and apoptosis of pancreatic carcinoma cells to provide a new prospect for the clinical treatment of pancreatic cancer.

## MATERIALS AND METHODS

### Materials

Quantum dots-RGD probe synthesized by School of Materials Science and Engineering, Shanghai University. Pancreatic cell line SW1990 purchased from Chinese Academy of Science. DEPC, Trizol, anhydrous ethanol, DAPI, MTT, DMSO, reactive oxygen species assay kit purchased from Beyotime institute of Biotechnology. Annexin V-FITC/PI purchased from invitrogen. RPMI 1640, 0.25% trypsin, fetal bovine serum, phosphate buffered saline purchased from Gibco. Reverse transcription-polymerase chain reaction (RT-PCR) assay kit purchased from Takara.

### Methods

**Quantum dots-RGD probe targeting research on pancreatic cancer cells:** For laser confocal micros-

copy, cells grown on 35-mm two imaging dishes (Cat no. P35G-0-14-C, Ashland, MA, United States) were washed with PBS and then were incubated at 37 °C in the presence of 5 nmol/L quantum dots-RGD probe and probe with excessive RGD 1  $\mu$ mol for 1 h. Afterward, cells were washed in ice-cold PBS, fixed by 4% paraformaldehyde and examined with simultaneous 543-nm excitation for laser confocal microscopy imaging. DAPI 2  $\mu$ g/mL was utilized for the nuclear labeling localization which was incubated dark at room temperature for 15 min.

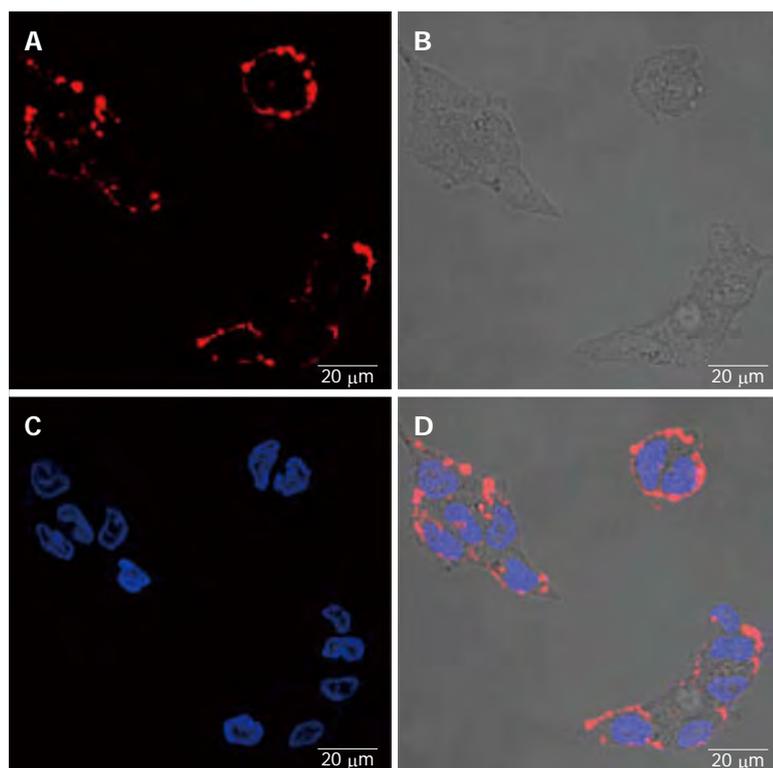
**Methyl thiazolyl tetrazolium assay test:** Cells grown on 96-well plates were divided into four groups, without QDs and light treated cells as blank control, pure light treated group, photosensitizer group, PDT group. Each group were set 10 double holes. Photosensitizer, PDT groups were incubated at 37 °C in the presence of 10 nmol/L Qds-RGD integrin-targeted probe. Pure light treated and PDT groups were treated with the wavelength of 690 nm laser, 20 J/cm<sup>2</sup> for 20 min. Absorbance (*A*) value were measured at the wavelength of 490 nm. Cells growth were observed after treated 24, 48 and 72 h. Cells relative inhibition rate (%) = (1 - the average *A* value of experimental group/the average *A* value of control group)  $\times$  100%.

**Morphologic changes in fluorescence microscopy:** After treated 48 h, cells were fixed by 4% paraformaldehyde and incubated with DAPI 2  $\mu$ g/mL. The morphological changes of cells were examined under fluorescence microscope.

**Cell cycle retardance and apoptosis observed by flow cytometry:** Each group were set 5 double holes. After treated 48 h, cells apoptosis and cell cycle retardance were observed by flow cytometry.

**Reactive oxygen species test:** Each group were set 3 double holes. After treated 48 h, DCFH-DA probe was applied to detect the expression of reactive oxygen species of four groups respectively.

**Relative gene expression by RT-PCR test:** According to the RT-PCR test kit procedure from Takara company, we observed the relative gene expression after PDT therapy. Gene primer sequences were synthesized by Shanghai Shengong. Myeloid cell leukemia-1 (Mcl-1) primer forward 5'-AAAGCCTGTCTGCCAAAT-3', reverse 5'-TATAAACCCACCACTCCC-3', product fragment 196 bp. Protein kinase B (Akt) primer: forward 5'-AAGCACCGCGTGACCATGAA-3', reverse 5'-TCT-TAATGTGCCCGTCCCTTG-3', product fragment 445 bp. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) primer: forward 5'-TGGCTAACT-GACCTGGAA-3', reverse 5'-GGAAACCTGGAGGC-TACT-3', product fragment 415 bp. Internal reference primer glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-CATGGTCTACACGTTCCAGT-3'. Re-



**Figure 1** The fluorescence expression of SW1990 cells with the imaging of quantum dots-arginine-glycine-aspartic acid probe. A: Quantum dots-arginine-glycine-aspartic acid probe imaging; B: Original image; C: 4',6-diamidino-2-phenylindole, dihydrochloride dyeing; D: Integrated image.

verse 5'-GGCTAAGCAGTTGGTGGTGC-3', product fragment 349 bp. PCR reaction condition: 94 °C 3 min predenaturation, 94 °C 30 s, 55 °C 30 s, 72 °C 1 min ( $\times$  35 cycles) 72 °C extend for 5 min. Afterwards, grayscale scanning analysis was made by means of gel imaging analysis system. mRNA expression of the target gene = average gray value of specimens target gene/average gray value of the same specimen GAPDH.

### Statistical analysis

Quantitative data in accordance with the normal distribution were analyzed using the One-way ANOVA and Student-Neuman-Keuls, otherwise using the non-parametric tests. SPSS 17.0 statistical software was used for analysis, and the significance level  $\alpha$  was set at 0.05.

## RESULTS

### Quantum dots-RGD probe targeting research on pancreatic cancer cells

The fluorescence expression of SW1990 cells with the imaging of QDS-RGD probe: QDS-RGD probe imaging, original image, DAPI dyeing, and integrated image (Figure 1).

The fluorescence imaging of SW1990 cells with QDS-RGD probe at the block of excess RGD peptide: QDS-RGD probe imaging, original image, DAPI dyeing, and integrated image (Figure 2).

### Methyl thiazolyl tetrazolium assay test

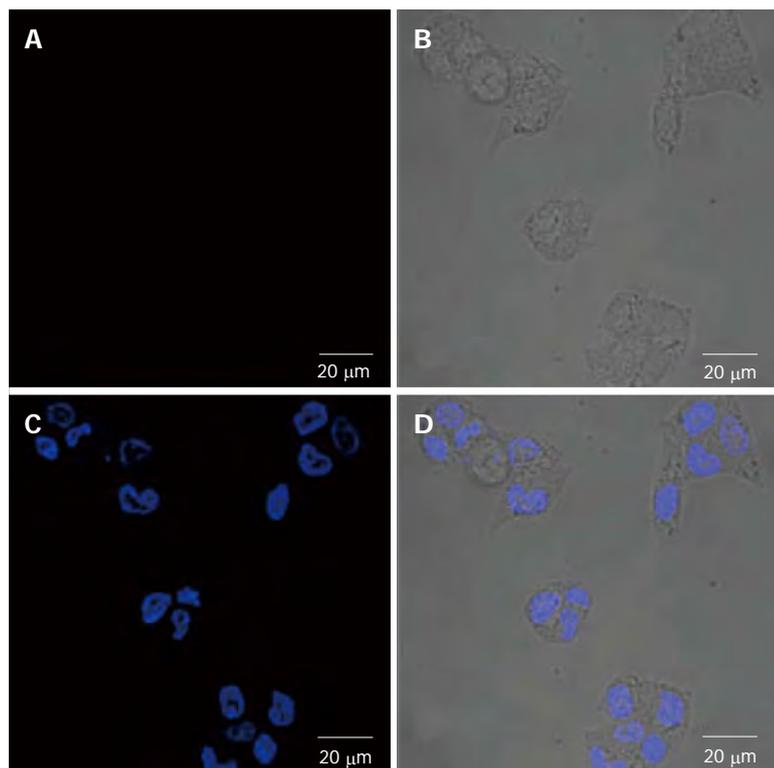
After 24, 48 or 72 h incubation, the PDT group cells growth inhibition rate was all higher than that of the control group, which makes statistically significant difference ( $F = 73.00, 85.10, 126.58, P < 0.01$ ). While compared with the control group, the cell growth inhibition rate of light group and photosensitizer group had no significant difference ( $P > 0.05$ ) (Table 1).

### Morphologic changes in fluorescence microscopy

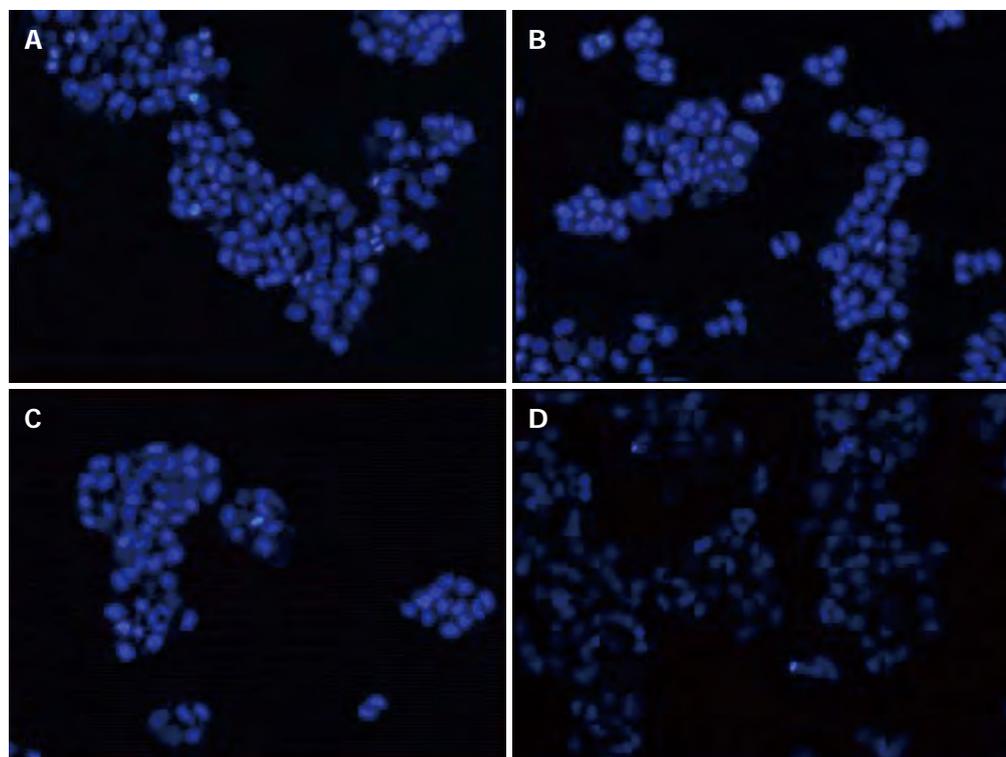
The cells nuclear morphologic changes in PDT group were corrugated under fluorescence microscopy. The nuclear of partial cells became staining deepen, bright blue, fuzzy and obviously pyknotic. With time prolonged, a large number of nuclear began to become dark, pyknotic and fragmentation. The remaining cells showed no significant change (Figure 3).

### Cell cycle retardance and apoptosis observed by flow cytometry

After treated for 48 h, the cell apoptotic rate of PDT group ( $17.86\% \pm 1.230\%$ ) was higher than the control ( $7.62\% \pm 1.219\%$ ), the light group ( $8.38\% \pm 0.277\%$ ) and the photosensitizer group ( $8.96\% \pm 0.673\%$ ), which made statistical difference ( $F = 130.617, P < 0.01$ , Figure 4). Compared to the control, PDT group had cell cycle  $G_0/G_1$  and S retardance ( $G_0/G_1$   $69.14\% \pm 2.63\%$  vs  $55.74\% \pm 2.82\%$ , S  $24.41\% \pm 2.67\%$  vs  $37.47\% \pm 0.74\%$ ,



**Figure 2** The fluorescence imaging of SW1990 cells with quantum dots-arginine-glycine-aspartic acid probe at the block of excess arginine-glycine-aspartic acid peptide. A: Quantum dots-arginine-glycine-aspartic acid probe imaging; B: Original image; C: 4',6-diamidino-2-phenylindole, dihydrochloride dyeing; D: Integrated image.

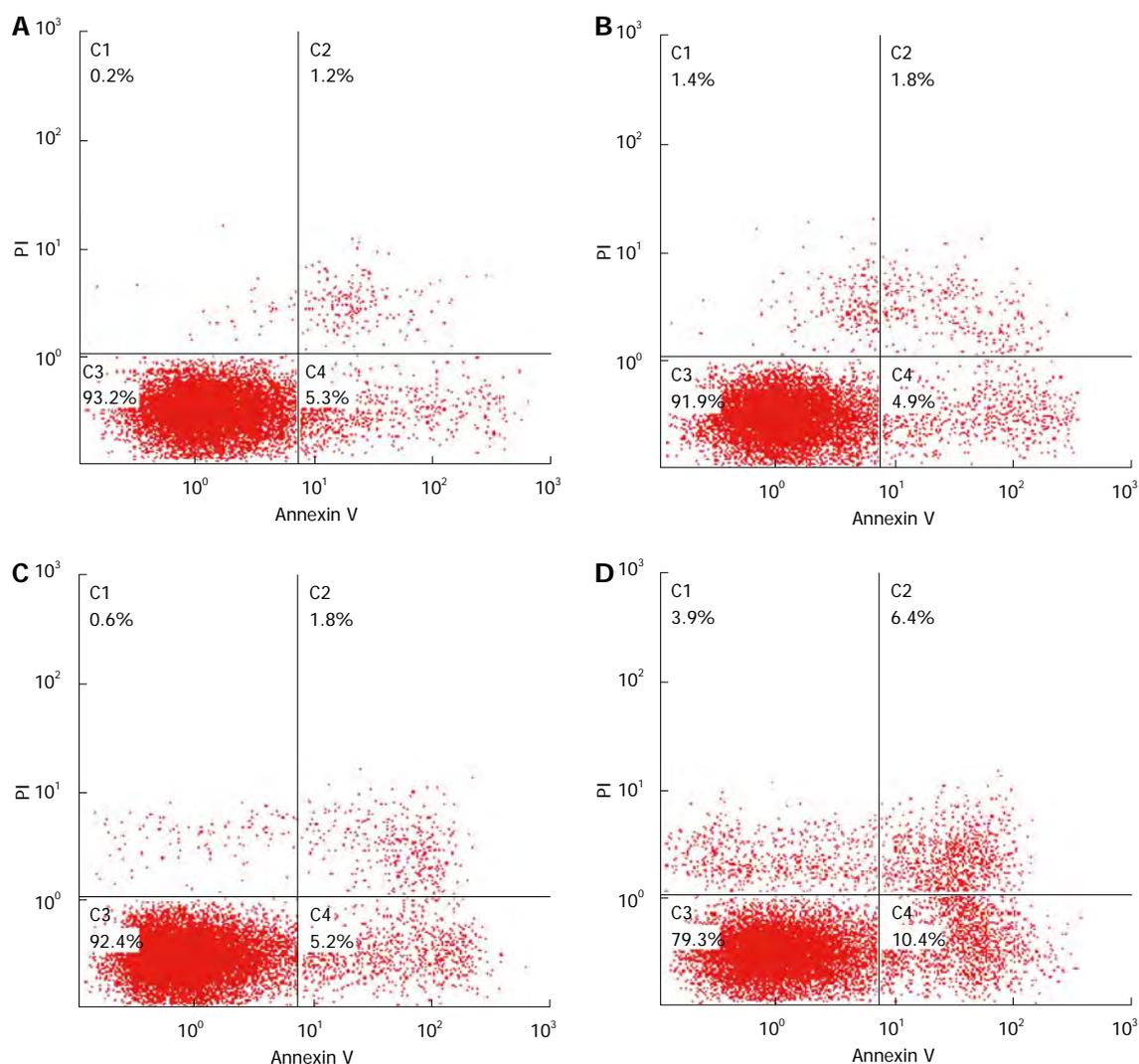


**Figure 3** The morphologic changes of cells observed under fluorescence microscope with the stain of DAPI after treated separately ( $\times 100$ ). A: Blank group; B: Light group; C: Quantum dots-arginine-glycine-aspartic acid group; D: Photodynamic therapy group.

**Table 1** The inhibition, and cell cycles effects of cells by methyl thiazolyl tetrazolium assay after treated separately

Treat grouping	24 h	48 h	72 h	G <sub>0</sub> /G <sub>1</sub>	S
Control group	0.213% ± 0.079%	0.216% ± 0.071%	0.274% ± 0.065%	55.74% ± 2.82%	37.47% ± 0.74%
Light group	0.217% ± 0.053%	0.242% ± 0.057%	0.281% ± 0.042%	53.73% ± 1.37%	37.88% ± 3.60%
Photosensitizer group	0.280% ± 0.061%	0.273% ± 0.055%	0.300% ± 0.050%	57.72% ± 0.91%	33.90% ± 3.39%
PDT group	0.566% ± 0.052% <sup>b</sup>	0.600% ± 0.062% <sup>b</sup>	0.651% ± 0.045% <sup>b</sup>	69.14% ± 2.63% <sup>b</sup>	24.41% ± 2.67% <sup>b</sup>

<sup>b</sup>*P* < 0.01 vs control group. PDT: Photodynamic therapy.



**Figure 4** The cell apoptosis of cells after treated separately. A: Control group; B: Light group; C: Quantum dots-arginine-glycine-aspartic acid group; D: Photodynamic therapy (PDT) group.

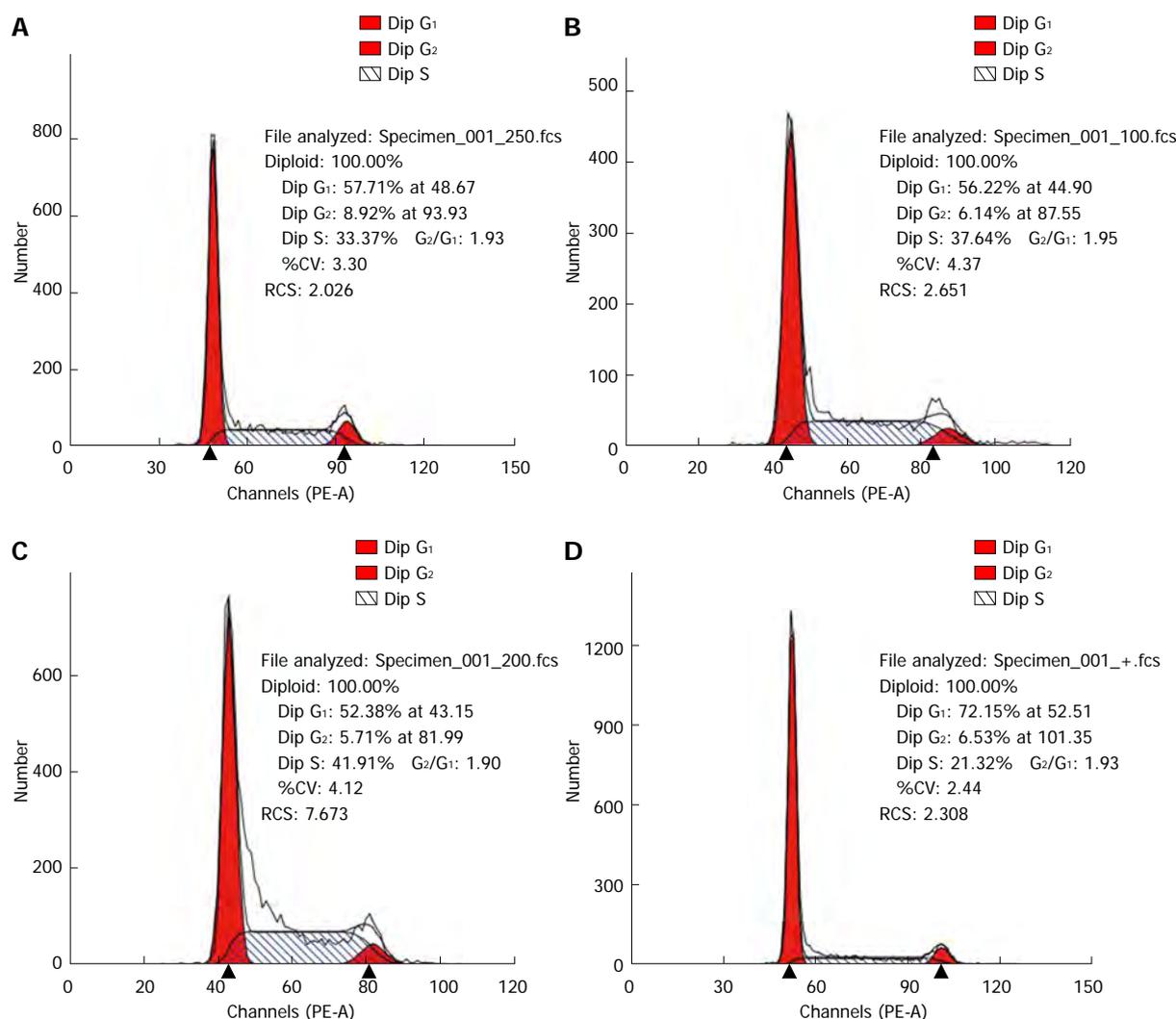
all *P* < 0.05, Figure 5 and Table 1).

### Reactive oxygen species test

Each group was observed five high fields and figured out the mean value of the cells. The cells of each HPF expressing fluorescence in PDT group ( $286 \pm 5.508$ ) were higher than the control ( $28 \pm 2.646$ ), the light group ( $34 \pm 2.082$ ) and the photosensitizer group ( $36 \pm 4.163$ ), which made statistical difference ( $F = 3262.559$ ,  $P < 0.01$ ) (Figure 6).

### Relative gene expression by RT-PCR test

The grayscale ratio of Akt mRNA and GADPH mRNA of the control, the light group, the photosensitizer group and PDT group were  $1.3319 \pm 0.0482$ ,  $1.3137 \pm 0.0100$ ,  $1.2900 \pm 0.0116$ ,  $0.6161 \pm 0.0032$ , respectively. The ratio of PDT group was lower than the others, which made statistical difference ( $F = 567.456$ ,  $P < 0.05$ ); The relative expression level of Mcl-1 was  $0.9864 \pm 0.0056$ ,  $1.0467 \pm 0.0248$ ,  $1.0187 \pm 0.0205$ ,  $0.3394 \pm 0.0451$ , respectively. The ratio of these two mRNA in PDT group were



**Figure 5** Cell cycle of cells after treated separately. A: Control group; B: Light group; C: Quantum dots-arginine-glycine-aspartic acid group; D: Photodynamic therapy group.

lower than the others, which made statistical difference ( $F = 446.817, P < 0.05$ ); the relative expression level of TRAIL was  $0.6221 \pm 0.0076, 0.6075 \pm 0.0072, 0.6189 \pm 0.0114, 0.7239 \pm 0.0017$ , respectively. The ratio of PDT group was higher than the others, which made statistical difference ( $F = 145.238, P < 0.05$ ) (Figure 7).

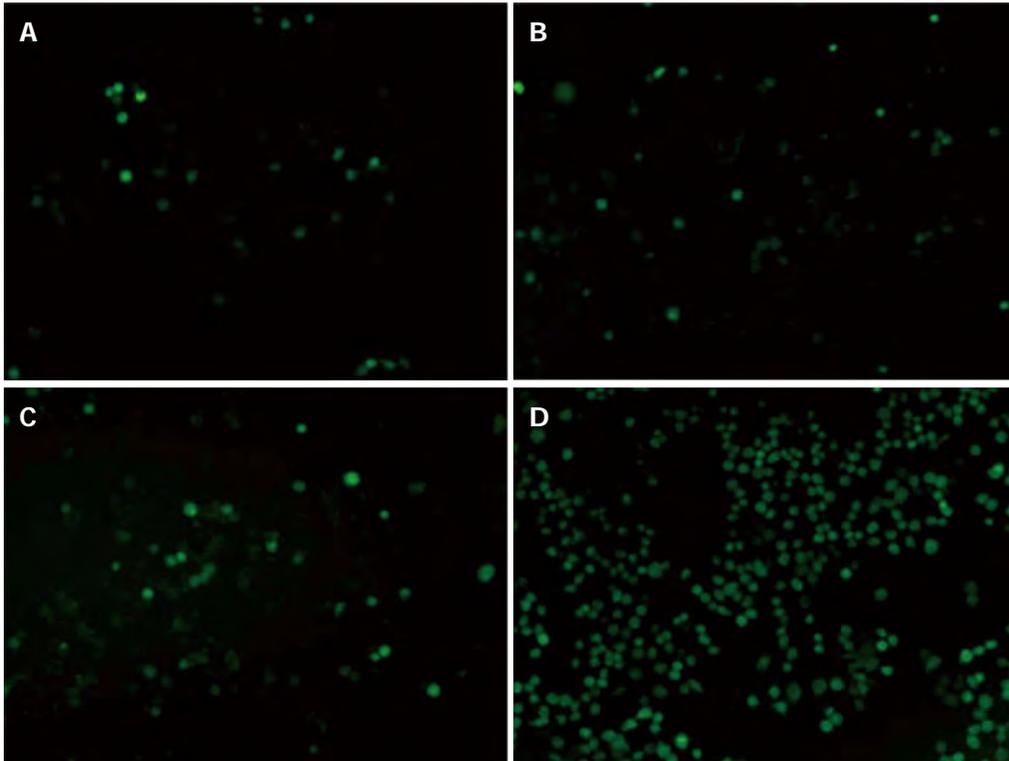
## DISCUSSION

Since its discovery in the early 1900s, photodynamic therapy has developed from an emerging cancer treatment to an Food and Drug Administration-approved therapy for different malignancies. Two major challenges of current PDT are the limited tissue penetration of excitation light and poor tumor-selectivity of the photosensitizer. To address these issues, we developed a multifunctional nano-construct consisting of Semiconductor quantum dots and RGD peptide. RGD peptide was coated on the surface of QDs to anchor the probe close to SW1990 cells, thereby facilitating tumor imaging and targeted therapy.

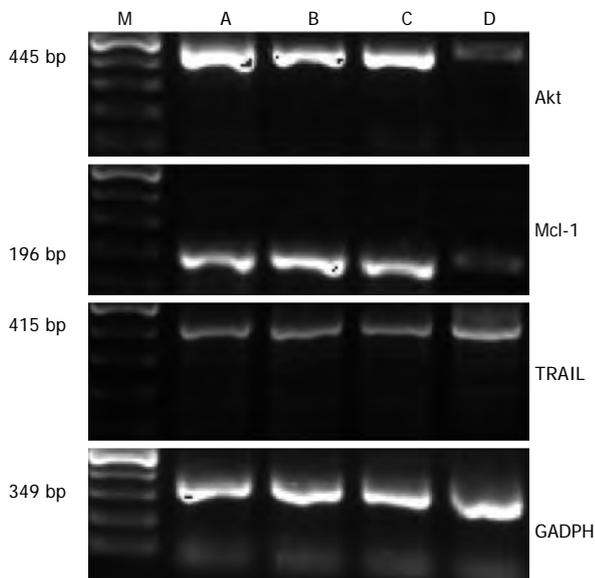
Semiconductor QDs, after surface modification to

render them water soluble and biocompatible, have a promising future in biomedical applications. QDs have size- and composition-adjustable fluorescence emission wavelengths, narrow emission bands, and very high levels of brightness and photostability. For *in vitro* studies, QDs have been used for cell labeling, fluorescence in situ hybridization, cell tracking, fluorescence resonance energy transfer, photodynamic therapy and many other applications<sup>[13-16]</sup>. QDs can serve as energy donors to conventional photosensitizers through FRET or interact directly with molecular oxygen *via* energy transfer mechanisms, to generate reactive <sup>1</sup>O<sub>2</sub> species that can be exploited for PDT<sup>[17,18]</sup>.

Members of the integrin family influence several aspects of tumor progression and metastasis, including cell survival, proliferation, and angiogenesis. The  $\alpha_v\beta_3$  integrin is the most prominent receptor class affecting tumor growth, tumor invasiveness, metastasis, tumor-induced angiogenesis, inflammation, osteoporosis, and rheumatoid arthritis. The  $\alpha_v\beta_3$  integrin is strongly expressed on tumor cells and activated endothelial cells. In contrast,



**Figure 6** Number of cells producing reactive oxygen species after treated separately. A: Control group; B: Light group; C: Quantum dots-arginine-glycine-aspartic acid group; D: Photodynamic therapy group.



**Figure 7** Expression of myeloid cell leukemia-1, protein kinase B and tumor necrosis factor-related apoptosis-inducing ligand mRNA in each group by reverse transcription-polymerase chain reaction analysis. A: Control group; B: Light group; C: Quantum dots-arginine-glycine-aspartic acid group; D: Photodynamic therapy group. Mcl-1: Myeloid cell leukemia-1; Akt: Protein kinase B; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

expression of  $\alpha_v\beta_3$  integrin is weak on resting endothelial cells and most normal tissues<sup>[8,9,19]</sup>. The  $\alpha_v\beta_3$  antagonists are being studied as anti-tumor and anti-angiogenic agents. A tripeptide sequence consisting of Arg-Gly-Asp

(RGD) has been identified as a recognition motif used by extracellular matrix proteins (vitronectin, fibrinogen, laminin, and collagen) to bind to a variety of integrins, including  $\alpha_v\beta_3$ .

Confocal microscopy demonstrated the enhanced tumor-selectivity of the nanoconstructs to cancer cells that overexpressed integrin receptor. The integrin-positive SW1990 cells were clearly visualized in fluorescence images from QDs-RGD binding. The fluorescence intensity correlated with the integrin expression level of the cell lines. This binding was blocked effectively by excessive 1  $\mu\text{mol/L}$  RGD. Unconjugated QDs showed no significant binding to the cell lines.

*In vitro*, the light-triggered PDT based on the nanoconstructs possessed remarkable therapeutic efficacy with tumor cell inhibition. The result of MTT showed that the PDT group cells growth inhibition rate was all higher than that of the control group, which made statistically significant difference ( $P < 0.01$ ). While dealt with quantum dots or light irradiation alone, the cells did not showed significant destruction ( $P > 0.05$ ). Apoptotic cells and morphologic changes could be found under optical microscope. The FCM revealed PDT group had more significant cell apoptosis rate compared to control cells ( $F = 130.617, P < 0.01$ ) and cell cycle  $G_0/G_1$  and S retardance ( $P < 0.05$ ) compared to control cells. All the results demonstrated that photodynamic therapy based on integrin targeted probe could really inhibit pancreatic cancer cell proliferation and induce apoptosis, which were similar to the results of Yu *et al*<sup>[20]</sup> and Liu *et al*<sup>[7]</sup>. Xie

*et al*<sup>[21]</sup> also reported that *in vivo* PDT combined with gemcitabine chemotherapy drugs could effectively inhibit the proliferation of pancreatic tumors and kill tumor tissue, which confirmed the inhibition and killing effect of PDT treatment in pancreatic cancer.

Akt is an important molecule involved in cell growth and invasion. *Bcl-2* gene family have close relationship with cell apoptosis, including Mcl-1 (apoptosis inhibition) and TRAIL (apoptosis promotion) among members of the family. In our study, the expression of Mcl-1 and Akt were down-regulated, while expression of TRAIL mRNA was up-regulated after SW1990 cells treated with PDT. All the changes revealed the direction to promoting apoptosis.

The literature indicated that reactive oxygen species (ROS) was the activator of mitochondrial damage and apoptosis, which is highly reactive and toxic to cell membranes, lysosomes, and mitochondria<sup>[22]</sup>. Our study showed that PDT group had more significant number of SW1990 cells producing reactive oxygen species compared to the control, which revealed that the PDT promoted pancreatic cancer cells generating large amounts of ROS resulting in apoptosis. It had been reported that cancer cells had lower ability to scavenge oxygen free radicals and were more sensitive to ROS than the normal<sup>[22]</sup>. Though pancreatic cancer cell growth inhibition could also occur with the treatment of laser or photosensitizer alone, the inhibition in PDT group was significantly enhanced. The results revealed that PDT could significantly change the redox state of the pancreatic cancer cells and enhance their level of oxidative stress, which were similar to the others. The literature indicated that the semiconductor nanoparticles were not internalized in the cell nuclei but in the cytoplasm<sup>[22]</sup>. Furthermore, the nanoparticles were internalized into the lysosomes. Lysosomes were the primary cellular targets of photodynamic therapy. The ruptured lysosomes release cathepsins B or L, which activate initiator caspases or inhibited the Bcl-2 family, which then promoted apoptosis *via* the mitochondria. The expression changes of apoptosis related genes, Bcl-2 and TRAIL, were consistent with the literature reported<sup>[22]</sup>. Therefore, with this study, we proved for the first time that the increased cell death by the integrin-targeted photodynamic therapy.

Furthermore, it had been reported that photodynamic therapy could kill the pancreatic tumor *in vitro*<sup>[20]</sup> and was also effective to improve the chemotherapy drug gemcitabine resistance<sup>[23]</sup>. *In vivo*, it had been reported that PDT and chemotherapy drugs could have synergistic effect on killing tumor. PDT was respected to be a new promising method because of broad application in pre-cancerous lesions and partial pancreas advanced lesions<sup>[24]</sup>.

In summary, these results indicate that the multifunctional nanoconstruct is a promising PDT agent for deep-seated tumor treatment and demonstrate a new paradigm for enhancing PDT efficacy. Photodynamic therapy can be an effective treatment of patients with pancreatic cancer, but more extensive preclinical and clinical trials are

needed for further improvement in the clinical application of PDT, especially in avoidance of complications during PDT.

## COMMENTS

### Background

A minimally invasive treatment capable of local destruction of tumor tissue with low morbidity may have a place in the treatment of pancreatic adenocarcinoma. The photodynamic therapy is a treatment that has high specificity, minimal invasiveness and good cosmetic outcome, which produces local necrosis of tissue with light after prior administration of a photosensitizing agent. They investigated the effects of photodynamic therapy with quantum dots-arginine-glycine-aspartic acid (RGD) probe as photosensitizer on the proliferation and apoptosis of pancreatic carcinoma cells to provide a new prospect for the clinical treatment of pancreatic cancer.

### Research frontiers

The photodynamic therapy is a treatment that has high specificity, minimal invasiveness and good cosmetic outcome, which produces local necrosis of tissue with light after prior administration of a photosensitizing agent. In the past few years, little studies have been reported in the literature about the photodynamic therapy (PDT) on pancreatic cancer treatment, especially for the targeted-PDT.

### Innovations and breakthroughs

Two major challenges of current photodynamic therapy (PDT) are the limited tissue penetration of excitation light and poor tumor-selectivity of the photosensitizer. To address these issues, authors developed a multifunctional nanoconstruct consisting of Semiconductor quantum dots and RGD peptide. RGD peptide was coated on the surface of quantum dots to anchor the probe close to SW1990 cells, thereby facilitating tumor imaging and targeted therapy.

### Applications

These results indicate that the multifunctional nanoconstruct is a promising PDT agent for deep-seated tumor treatment and demonstrate a new paradigm for enhancing PDT efficacy. Photodynamic therapy can be an effective treatment of patients with pancreatic cancer, but more extensive preclinical and clinical trials are needed for further improvement in the clinical application of PDT, especially in avoidance of complications during PDT.

### Peer review

This is a good descriptive study in which authors evaluate the effect of photodynamic therapy with quantum dots-RGD probe as photosensitizer on the proliferation and apoptosis of pancreatic carcinoma cells. The results are interesting and suggest that significantly inhibits cell proliferation and increases apoptosis in SW1990 cells, which provide a new prospect for the clinical treatment of pancreatic cancer.

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## Characteristics and prognosis of gastric cancer in patients aged $\geq 70$ years

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### Abstract

**AIM:** To elucidate the prognostic value of age for gastric cancer and identify the optimal treatment for elderly gastric cancer patients.

**METHODS:** We enrolled 920 patients with gastric cancer who underwent gastrectomy between January 2003 and December 2007 in our center. Patients were categorized into three groups: younger group (age < 50 years), middle-aged group (50-69 years), and elderly group ( $\geq 70$  years). Clinicopathological features were compared among the three groups and potential prognostic factors were analyzed. The log-rank test was

used to assess statistical differences between curves. Independent prognostic factors were identified by the Cox proportional hazards regression model. Stratified analysis was used to investigate the impact of age on survival at each stage. Cancer-specific survival was also compared among the three groups by excluding deaths due to reasons other than gastric cancer. We analyzed the potential prognostic factors for patients aged  $\geq 70$  years. Finally, the impact of extent of lymphadenectomy and postoperative chemotherapy on survival for each age group was evaluated.

**RESULTS:** In the elderly group, there was a male predominance. At the same time, cancers of the upper third of the stomach, differentiated type, and less-invasive surgery were more common than in the younger or middle-aged groups. Elderly patients were more likely to have advanced tumor-node-metastasis (TNM) stage and larger tumors, but less likely to have distant metastasis. Although 5-year overall survival (OS) rate specific to gastric cancer was not significantly different among the three groups, elderly patients demonstrated a significantly lower 5-year OS rate than the younger and middle-aged patients (elderly vs middle-aged vs younger patients = 22.0% vs 36.6% vs 38.0%, respectively). In the TNM-stratified analysis, the differences in OS were only observed in patients with II and III tumors. In multivariate analysis, only surgical margin status, pT4, lymph node metastasis, M1 and sex were independent prognostic factors for elderly patients. The 5-year OS rate did not differ between elderly patients undergoing D1 and D2 lymph node resection, and these patients benefited little from chemotherapy.

**CONCLUSION:** Age  $\geq 70$  years was an independent prognostic factor for gastric cancer after gastrectomy. D1 resection is appropriate and postoperative chemotherapy is possibly unnecessary for elderly patients with gastric cancer.

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**Key words:** Gastric carcinoma; Elderly patients; Prognosis; Lymphadenectomy; Chemotherapy

**Core tip:** Few studies have compared the characteristics and prognosis of gastric cancer among younger, middle-aged and elderly patients. Elderly patients have distinctive properties, and we have to treat them individually with particular care. We found that age  $\geq 70$  years was an independent prognostic factor for patients with gastric cancer after gastrectomy and these patients had distinctive characteristics of male predominance, larger tumor size, more histological differentiation, higher number of tumors located in the upper third of the stomach, and advanced tumor-node-metastasis stage, but less distant metastasis compared to younger and middle-aged patients.

Liang YX, Deng JY, Guo HH, Ding XW, Wang XN, Wang BG, Zhang L, Liang H. Characteristics and prognosis of gastric cancer in patients aged  $\geq 70$  years. *World J Gastroenterol* 2013; 19(39): 6568-6578 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6568.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6568>

## INTRODUCTION

The population of China is growing both larger and older. According to the National Bureau of Statistics of China (NBSC, 2011), the population of China reached 1.35 billion at the end of 2011, making it the most populous country in the world, and the number of people aged  $\geq 65$  years has risen to 118 million, or approximately 8.87% of the total population compared to 6.96% in 2000. With the aging of the population, the number of patients aged  $\geq 70$  years with gastric cancer is increasing in China. It has been reported that surgery is safe and the surgical outcome is better compared with the best supportive care in elderly gastric cancer patients<sup>[1,2]</sup>. However, it is still unclear whether surgical outcome in elderly patients differs from that in younger patients. In Japan, treatment guidelines for gastric cancer have been issued, and a standard therapeutic strategy for gastric cancer by stage has been established. Gastrectomy with D2 lymph node dissection has been increasingly regarded as the standard surgical procedure for most patients with operable gastric cancer. For elderly patients, it is not established whether these therapeutic strategies are suitable and controversy still exists. Previous studies have compared outcomes between elderly and younger patients with gastric cancer<sup>[3,4]</sup>. However, gastric cancer in younger patients also has distinctive properties.

In the present study, we compared the clinicopathological characteristics and surgical outcomes of gastric cancer among elderly patients ( $\geq 70$  years), middle-aged patients (50-69 years), and younger patients ( $< 50$  years).

Our ultimate aim was to identify the optimal treatment for elderly patients with gastric cancer.

## MATERIALS AND METHODS

### Patients

We reviewed surgical and pathological data of 920 patients with gastric cancer who had undergone gastrectomy with lymph node dissection, who were followed up between January 2003 and December 2007 at Tianjin Medical University Cancer Institute and Hospital. All the patients had histologically confirmed gastric adenocarcinoma. Patients who had previously undergone gastric surgery or had received neoadjuvant chemotherapy were excluded. There were 659 men (71.6%) and 261 women (28.4%), with a median age of 62 years (age range: 20-89 years). All the patients were categorized into the following three groups: younger group ( $< 50$  years old, 166 patients), middle-aged group (50-69 years old, 481 patients) and elderly group ( $\geq 70$  years, 273 patients).

### Surgical treatment and perioperative management

All the patients underwent gastrectomy with D1 or D2 lymph node dissection. The choice of surgical procedure of reconstruction was made according to the surgeon's preference. Resection margin was detected by histological examination. Negative resection margin was defined as microscopic complete resection, without residual cancer cells in the margin. Positive resection margin was defined as tumor cells  $< 1$  mm from the cut edge or residual cells in the margin. Postoperative adjuvant chemotherapy was implemented according to the tumor stage, physical condition and willingness of the patient. Chemotherapeutics consisted of 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX-6). Radiotherapy was not used in the present study.

### Evaluation of clinicopathological variables and survival

Clinicopathological features studied included sex, age, tumor location, tumor size, Borrmann type, histology, surgical margin status, extranodal metastasis, depth of invasion, lymph node metastasis, distant metastasis, tumor-node-metastasis (TNM) stage, extent of lymphadenectomy, type of gastrectomy, and postoperative chemotherapy. The tumors were staged according to the Union for International Cancer Control TNM classification system, 7<sup>th</sup> edition, and lymphadenectomy and lymph node stations were defined according to the 3<sup>rd</sup> English Edition of the Japanese Classification of Gastric Carcinoma. Tumors were classified into two groups based on histology: differentiated type, including papillary, well or moderately differentiated adenocarcinoma; and undifferentiated type, including poorly differentiated or undifferentiated adenocarcinoma, signet ring cell carcinoma and mucinous carcinoma.

### Follow-up

All the patients were followed every 3 mo until 2 years

after surgery, then every 6 mo for up to 5 years, and then every year or until death. Physical examination, laboratory tests, imaging and endoscopy were performed at every visit. The median follow-up was 26 mo (range: 1-103 mo), and the last follow-up date was December 25, 2012. The overall survival rate was calculated from the day of surgical resection until time of death or final follow-up.

### Statistical analysis

Categorical variables were analyzed by means of the  $\chi^2$  or Fisher's exact test. Overall survival curves were calculated using the Kaplan-Meier method based on the length of time between primary surgical treatment and final follow-up or death. The log-rank test was used to assess statistical differences between curves. Independent prognostic factors were identified by Cox proportional hazards regression model.  $P < 0.050$  was considered statistically significant. The statistical analysis was performed using SPSS version 17.0 (Chicago, IL, United States).

## RESULTS

### Clinicopathological features

Of the 920 patients who underwent gastrectomy, 793 patients achieved a negative resection margin (241 elderly, 415 middle-aged and 137 younger patients), and 127 patients had a positive resection margin (32 elderly, 66 middle-aged and 29 younger patients). Of all the patients, 402 (43.7%) underwent D2 lymph nodes dissection, and 518 (56.3%) D1 dissection, including 65 (7.1%) patients who accepted palliative surgery without formal lymph node dissection because of distant metastasis. Six hundred and seventy-one (72.9%) patients accepted surgery alone and 249 (27.1%) surgery plus postoperative chemotherapy with FOLFOX-6.

All the patients were divided into three categories according to their age (Table 1). The mean age was 42.8 years in the younger group, 60.1 years in the middle-aged group, and 74.1 years in the elderly group. There were no significant differences in Borrmann type, extranodal metastasis, surgical margin status, depth of invasion, and lymph node metastasis among the three groups. In the elderly group, there was a male predominance, and cancers of the upper third of the stomach and differentiated type, and less-invasive surgery were more common than in the younger and middle-aged groups. The elderly patients were more likely to have advanced TNM stage and larger tumors, but less likely to have distant metastasis and undergo postoperative chemotherapy. The rate of distant metastasis was 10.2% in the younger group, 8.1% in the middle-aged group, and 3.3% in the elderly group. Although there were no significant differences in type of distant metastasis, elderly patients were more likely to have liver metastasis, but less likely to have peritoneal metastasis than younger or middle-aged patients.

### Prognostic value of age in gastric cancer

The results of univariate and multivariate analysis of all

920 patients are shown in Table 2. Surgical margin status, pT<sub>4</sub>, N stage, M1, extent of lymphadenectomy, postoperative chemotherapy and age  $\geq 70$  years (hazard ratio: 1.487,  $P = 0.003$ ) remained as independent prognostic factors for overall survival (OS). Patients aged  $\geq 70$  years demonstrated a significantly lower 5-year OS rate than the younger and middle-aged patients (elderly *vs* middle-aged *vs* younger patients, 22.0% *vs* 36.6% *vs* 38.0%, respectively) (Figure 1). In the TNM-stratified analysis, the differences in OS were only observed in patients with TNM stage II and III cancer (Table 3 and Figure 2). However, when deaths caused by factors other than gastric cancer were excluded, there were no significant differences in cancer-specific survival among the three groups (Figure 3).

### Survival of patients aged $\geq 70$ years

Survival analysis of the elderly patients is shown in Table 4. Sex, tumor size, histology, extranodal metastasis, surgical margin status, pT<sub>4</sub>, lymph node metastasis, M1 and type of gastrectomy were found to be prognostic factors in the univariate analysis, while only surgical margin status, pT<sub>4</sub>, lymph node metastasis, M1 and sex were independent prognostic factors in the multivariate analysis. For patients aged  $\geq 70$  years, women tended to have a significantly higher 5-year OS than men (29.3% *vs* 20.0%,  $P = 0.045$ ). Although the patients who underwent D2 resection had better survival than those with D1 resection, there was no significant difference in OS between D1 and D2 resection for the elderly patients. In the stratified analysis, chemotherapy was a prognostic factor for the younger and middle-aged patients, but not for the elderly patients (Table 5 and Figure 4).

## DISCUSSION

The number of elderly patients with gastric cancer is rapidly increasing with an aging population. With regard to elderly, no clear-cut distinction exists. Previous studies used 65, 70, 75, 80 and 85 years as thresholds<sup>[1-7]</sup>. Data from NBSC show that the average life span in China was 72.38 years for men and 77.37 years for women in 2010, and patients aged  $\geq 65$  years accounted for 8.87% of the total population. Taking into consideration that the majority of elderly patients with gastric cancer are male, we used 70 years as a threshold, which is close to the average life span of Chinese men. Many elderly patients with gastric cancer also suffer from comorbid diseases such as hypertension, diabetes mellitus, ischemic heart disease, brain infarction, or renal dysfunction. Therefore, we have to treat elderly patients individually with particular care. Gastric cancer in elderly patients actually presents a distinctive entity with specific clinicopathological characteristics.

The current study showed that the distinguishing characteristics in the elderly gastric cancer patients included male predominance, more histologically differentiated type, higher rate of tumors located in the upper third of the stomach, larger tumor size, more advanced

Table 1 Case characteristics *n* (%)

Characteristics	Age (yr)			$\chi^2$	P value
	< 50 ( <i>n</i> = 166)	50-69 ( <i>n</i> = 481)	≥ 70 ( <i>n</i> = 273)		
Age (mean ± SD)	42.8 ± 5.8	60.1 ± 5.5	74.1 ± 3.4		
Gender				32.504	< 0.001
Female	76 (45.8)	127 (26.4)	58 (21.2)		
Male	90 (54.2)	354 (73.6)	215 (78.8)		
Tumor location				39.900	< 0.001
Lower 1/3	83 (50.0)	201 (41.8)	88 (40.4)		
Middle 1/3	31 (18.7)	87 (18.1)	48 (17.6)		
Upper 1/3	21 (12.7)	143 (29.7)	106 (38.8)		
2/3 or more	31 (18.7)	50 (10.4)	31 (11.4)		
Tumor location				34.186	< 0.001
Upper 1/3	21 (12.7)	143 (29.7)	106 (38.8)		
Non-upper 1/3	145 (87.3)	338 (70.3)	167 (61.2)		
Tumor size				13.589	0.001
< 5 cm	79 (47.6)	209 (43.5)	87 (31.9)		
≥ 5 cm	87 (52.4)	272 (56.5)	186 (68.1)		
Tumor size, cm (mean ± SD)	5.640 ± 3.172	5.552 ± 2.810	6.304 ± 3.644	5.035	0.007
Borrmann type				2.443	0.296
I / II	47 (28.3)	166 (34.5)	95 (34.8)		
III / IV	119 (71.7)	315 (65.5)	178 (65.2)		
Histology				39.366	< 0.001
Differentiated	24 (14.5)	139 (28.9)	116 (42.5)		
Undifferentiated	142 (85.5)	342 (71.1)	157 (57.5)		
Extranodal metastasis				4.733	0.094
Negative	121 (72.9)	378 (78.6)	223 (81.7)		
Positive	45 (27.1)	103 (21.4)	50 (18.3)		
Surgical margin status				2.873	0.238
Negative	137 (82.5)	415 (86.3)	241 (88.3)		
Positive	29 (17.5)	66 (13.7)	32 (11.7)		
Depth of invasion				5.457	0.487
pT <sub>1</sub>	4 (2.4)	12 (2.5)	6 (2.2)		
pT <sub>2</sub>	22 (13.3)	49 (10.2)	22 (8.1)		
pT <sub>3</sub>	5 (3.0)	30 (6.2)	17 (6.2)		
pT <sub>4</sub>	135 (81.3)	390 (81.1)	228 (83.5)		
Lymph node metastasis				5.325	0.503
pN <sub>0</sub>	62 (37.3)	170 (35.5)	87 (31.9)		
pN <sub>1</sub>	26 (15.7)	83 (17.3)	51 (18.7)		
pN <sub>2</sub>	32 (19.3)	108 (22.5)	73 (26.7)		
pN <sub>3</sub>	46 (27.7)	120 (24.9)	62 (22.7)		
Distant metastasis				9.251	0.010
M <sub>0</sub>	149 (89.8)	442 (91.9)	264 (96.7)		
M <sub>1</sub>	17 (10.2)	39 (8.1)	9 (3.3)		
Types of distant metastasis				5.502	0.240
Liver metastasis	5 (29.4)	19 (48.7)	5 (55.6)		
Peritoneal metastasis	12 (70.6)	18 (46.2)	3 (33.3)		
Other distant metastasis	0 (0.0)	2 (5.1)	1 (11.1)		
TNM stage				13.270	0.039
I	19 (11.4)	43 (8.9)	21 (7.7)		
II	45 (27.1)	135 (28.1)	72 (26.4)		
III	85 (51.2)	264 (54.9)	171 (62.6)		
IV	17 (10.2)	39 (8.1)	9 (3.3)		
Chemotherapy				48.852	< 0.001
Yes	71 (42.8)	142 (29.5)	36 (13.2)		
No	95 (57.2)	339 (70.5)	237 (86.8)		
Type of gastrectomy				7.466	0.024
Subtotal	112 (67.5)	361 (75.1)	216 (79.1)		
Total	54 (32.5)	120 (24.9)	57 (20.9)		
Extent of lymphadenectomy				19.735	< 0.001
D <sub>2</sub>	89 (53.6)	222 (46.2)	91 (33.3)		
D <sub>1</sub>	77 (46.4)	259 (53.8)	182 (66.7)		

TNM stage, and less distant metastasis compared to the younger and middle-aged patients. Also in the elderly group, subtotal gastrectomy and D1 resection were more frequently performed, while few patients underwent

postoperative chemotherapy.

Many studies have shown a male predominance in elderly gastric cancer patients<sup>[8,9]</sup>, and in younger patients (< 40 years), the sex ratio has been reported to be ap-

**Table 2** Survival analysis of all patients with gastric cancer after surgery

Characteristics	n (%)	5-yr OS	MST (mo)	Univariate analysis		Multivariate analysis	
				$\chi^2$	P (log-rank)	Hazard ratio (95%CI)	P value
Gender				0.165	0.685		
Male	659 (71.6)	32.00%	26				
Female	261 (28.4)	33.70%	27				
Age (yr)				21.067	< 0.000		
< 50	166 (18.0)	38.00%	32			1 (ref)	
50-69	481 (52.3)	36.60%	28			1.107 (0.881, 1.391)	0.383
≥ 70	273 (29.7)	22.00%	20			1.487 (1.149, 1.924)	0.003
Tumor location				37.996	< 0.001		
Lower 1/3	372 (40.4)	40.10%	36			1 (ref)	
Middle 1/3	166 (18.0)	30.10%	18			1.129 (0.881, 1.446)	0.339
Upper 1/3	270 (29.3)	29.60%	25			1.063 (0.864, 1.309)	0.564
2/3 or more	112 (12.2)	17.90%	16			1.311 (0.985, 1.744)	0.064
Tumor size				51.052	< 0.001		
< 5 cm	375 (40.8)	45.10%	42			1 (ref)	
≥ 5 cm	545 (59.2)	23.90%	20			1.185 (0.994, 1.413)	0.058
Borrmann type				9.588	0.002		
I / II	308 (33.5)	38.30%	33			1 (ref)	
III / IV	612 (66.5)	29.60%	23			1.171 (0.980, 1.399)	0.082
Histology				13.994	< 0.001		
Differentiated	279 (30.3)	41.20%	34			1 (ref)	
Undifferentiated	641 (69.7)	28.70%	23			1.201 (0.996, 1.449)	0.055
Extranodal metastasis				61.626	< 0.001		
Negative	722 (78.5)	37.10%	31			1 (ref)	
Positive	198 (21.5)	15.70%	15			1.164 (0.962, 1.409)	0.119
Surgical margin status				101.241	< 0.001		
Negative	793 (86.2)	36.70%	31			1 (ref)	
Positive	127 (13.8)	6.30%	11			1.705 (1.357, 2.142)	< 0.001
Depth of invasion				67.084	< 0.001		
pT <sub>1</sub>	22 (2.4)	86.40%	69			1 (ref)	
pT <sub>2</sub>	93 (10.1)	61.30%	61			3.048 (0.937, 9.918)	0.064
pT <sub>3</sub>	52 (5.7)	46.20%	47			3.188 (0.965, 10.526)	0.057
pT <sub>4</sub>	753 (81.8)	26.40%	21			4.580 (1.431, 14.200)	0.010
Lymph node metastasis				243.605	< 0.001		
pN <sub>0</sub>	319 (34.7)	57.70%				1 (ref)	
pN <sub>1</sub>	160 (17.4)	31.90%	24			1.713 (1.327, 2.211)	< 0.001
pN <sub>2</sub>	213 (23.2)	23.50%	23			1.918 (1.514, 2.429)	< 0.001
pN <sub>3</sub>	228 (24.8)	6.10%	13			3.268 (2.572, 4.151)	< 0.001
Distant metastasis				89.428	< 0.001		
M <sub>0</sub>	855 (92.9)	34.70%	29			1 (ref)	
M <sub>1</sub>	65 (7.1)	3.10%	8			1.817 (1.339, 2.465)	< 0.001
Chemotherapy				17.080	< 0.001		
Yes	249 (27.1)	39.80%	37			1 (ref)	
No	671 (72.9)	29.80%	22			1.383 (1.144, 1.673)	0.001
Type of gastrectomy				40.899	< 0.001		
Subtotal	689 (73.9)	36.90%	31			1 (ref)	
Total	231 (26.1)	19.50%	16			1.170 (0.944, 1.450)	0.151
Extent of lymphadenectomy				4.060	0.044		
D <sub>2</sub>	402 (43.7)	36.60%	28			1 (ref)	
D <sub>1</sub>	518 (56.3)	29.30%	24			1.192 (1.005, 1.414)	0.043

Ref: Reference category; OS: Overall survival; MST: Median survival time.

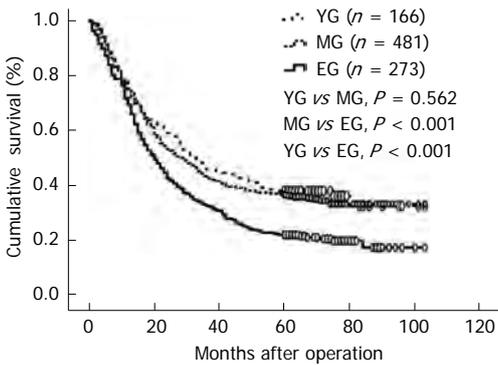
proximately 1:1<sup>[10]</sup>. Our findings are consistent with these reports. The sexual imbalance may reflect a more frequent and prolonged exposure of elderly male patients to environmental carcinogens. We also found that the proportion of the histologically differentiated type cancer increased with aging, from 14.5% in the younger patients to 42.5% in the elderly. Some studies concluded that gastric carcinoma in elderly patients may principally develop as well-differentiated lesions that progress to poorly differentiated ones, whereas in younger patients, most gastric carcinoma emerges as poorly differentiated type

at an early phase<sup>[11,12]</sup>. This may also be attributed to the fact that younger patients are more likely to have distant metastasis. Although many studies have demonstrated that gastric cancer in elderly patients was predominantly localized in the lower third of the stomach<sup>[8,11,12]</sup>, some researchers reported that cancer involving the upper third of the stomach was more common in elderly than in younger patients<sup>[13,14]</sup>. In our study, only 28.6% of tumors were located in the lower third of the stomach in elderly patients, and the ratio of upper-third tumors increased from 12.7% in the younger patients to 38.8% in the el-

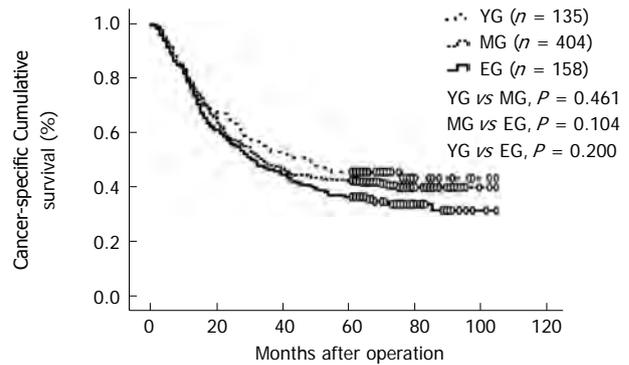
**Table 3** Survival analysis of younger, middle-aged and elderly patients stratified by tumor-node-metastasis stage

TNM	Younger (< 50 yr)			Middle-aged (50-69 yr)			Elderly (≥ 70 yr)			$\chi^2$	P value
	n	5-yr OS	MST (mo)	n	5-yr OS	MST (mo)	n	5-yr OS	MST (mo)		
I	19	78.90%	68	43	81.40%	64	21	66.70%	66	1.200	0.594
II	45	53.30%	60	135	60.00%	61	72	33.30%	39	13.024	0.001
III	85	27.10%	24	264	22.30%	20	171	12.90%	16	11.874	0.003
IV	17	5.90%	10	39	2.60%	8	9	0.00%	6	2.909	0.233

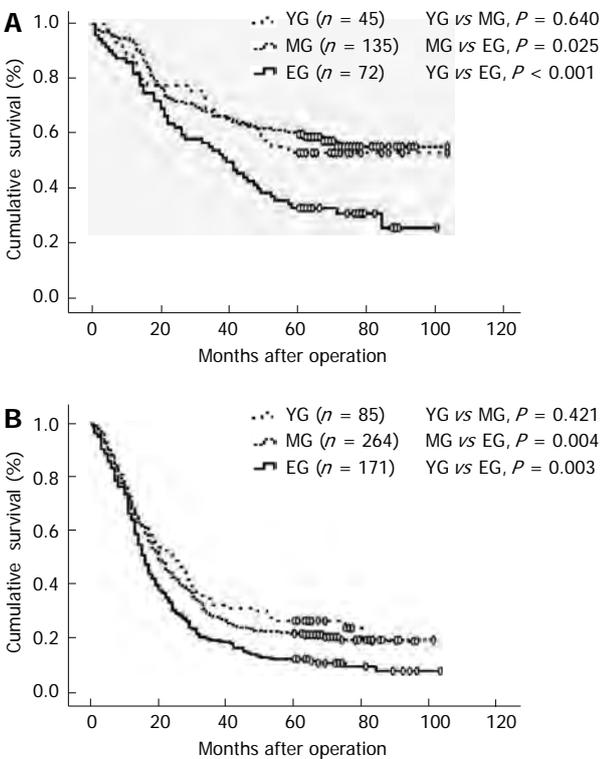
MST: Median survival time; TNM: Tumor-node-metastasis; OS: Overall survival.



**Figure 1** Overall survival curves for all patients grouped by age. Patients aged ≥ 70 years demonstrated a significantly lower 5-year OS rate than the younger and middle-aged patients (elderly vs middle-aged vs younger patients, 22.0% vs 36.6% vs 38.0%, respectively). EG: Elderly group; MG: Middle-aged group; YG: Younger group.



**Figure 3** Cancer-specific survival of each age group. When deaths caused by factors other than gastric cancer were excluded, there were no significant differences in cancer-specific survival among the three age groups. EG: Elderly group; MG: Middle-aged group; YG: Younger group.



**Figure 2** Overall survival curves for patients with tumor-node-metastasis II and III cancer. In the tumor-node-metastasis (TNM)-stratified analysis, the differences in overall survival (OS) were only observed in patients with TNM stage II and III cancer. A: Patients with II cancer; B: Patients with III cancer. EG: Elderly group; MG: Middle-aged group; YG: Younger group.

elderly patients. It is possible that the risk of developing carcinoma in the upper third of the stomach increases with advancing age. Previous reports have shown no significant difference in tumor stage between elderly and younger or middle-aged patients<sup>[15-17]</sup>. Although in the present study there were no significant differences in the depth of invasion and lymph node metastasis among the three groups, the elderly patients were more likely to have advanced TNM stage. The ratio of stage III cancer was 62.6% in the elderly patients compared to 51.2% in the younger patients. Usually the symptoms of gastric cancer are not obvious in elderly patients, which may result in delayed diagnosis. Thus, it is easier for advanced tumor stage and larger tumor size to develop in elderly than younger and middle-aged patients.

It has been reported that surgery is safe and surgical outcome is better compared with the best supportive care in elderly patients with gastric cancer<sup>[1,2]</sup>. Limited operation is predominant because total gastrectomy and D2 resection in elderly patients are associated with higher rates of postoperative morbidity and mortality compared to subtotal gastrectomy and D1 resection<sup>[15,18]</sup>. In the present study, subtotal gastrectomy and D1 resection were more frequently performed in the elderly patients. However, the long-term outcome of elderly patients is still controversial after limited operation.

Many studies have specifically compared the long-term outcome of gastric cancer in elderly patients with that in younger or middle-aged patients. Some found no significant difference in survival between them<sup>[15,19]</sup>. How-

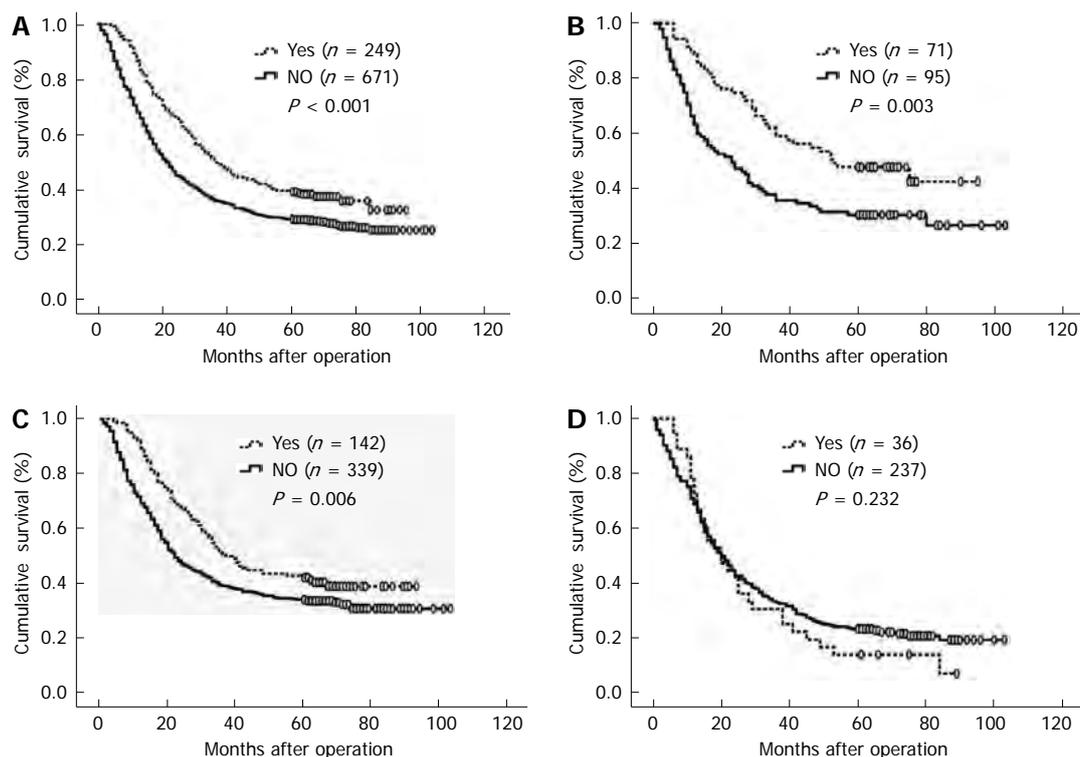


Figure 4 Overall survival curves for patients grouped by chemotherapy. In the age-stratified analysis, chemotherapy was a prognostic factor for the younger and middle-aged patients, but not for the elderly patients. A: All patients; B: Younger group; C: Middle-aged group; D: Elderly group.

ever, most studies confirmed the prognosis of elderly patients was poorer than that of younger and middle-aged patients<sup>[9,16,17]</sup>. Our results are consistent with most of those reports. Patients aged  $\geq 70$  years had a significantly lower 5-year OS rate than younger and middle-aged patients. In general, the poor prognosis of elderly patients can be attributed to the delay in diagnosis and advanced tumor stage<sup>[9,20]</sup>. However, in TNM-stratified analysis, such differences were still observed in stage II and III cancer. In multivariate analysis, age was an independent prognostic factor, as well as surgical margin status, pT<sub>4</sub>, lymph node metastasis, M1, chemotherapy and the extent of lymphadenectomy. It has been reported that as patients age, they have a reduced ability to tackle cancer growth, which may lead to poorer prognosis of elderly patients<sup>[21]</sup>. In our study, when deaths caused by other comorbid diseases and malignancies were excluded, there were no significant differences in cancer-specific survival among the three groups. This result agrees with previous studies<sup>[8,17,22,23]</sup>. According to our results, the poorer prognosis of the elderly patients with gastric cancer was due to other comorbid diseases and malignancies rather than gastric cancer itself. To improve the outcome of gastric cancer in elderly patients, we should pay attention to treating other comorbid diseases and malignancies in addition to providing adequate treatment for gastric cancer itself.

Elderly patients have distinguishing characteristics and prognosis from younger and middle-aged ones, thus, it is necessary to elucidate prognostic factors that influ-

ence OS in elderly patients. In the present study, sex, surgical margin status, pT<sub>4</sub>, lymph node metastasis and M1 disease were found to be independent prognostic factors for elderly patients with gastric cancer. Some studies have reported better prognosis for women than men<sup>[24,25]</sup>. Usually, women have a longer life-span than men. In 2005, life expectancy was 2.4 years longer for women than men in India, 3.2 years longer in China, 3.8 years longer in Indonesia, and 7.4 years longer in Japan<sup>[26]</sup>. This may account for the better prognosis of female elderly patients with gastric cancer. Depth of invasion, lymph node status and distant metastasis have been proven to be the most powerful independent prognostic factors for gastric cancer. However, few studies have specifically evaluated the prognostic value of these factors in elderly patients. Yokota *et al.*<sup>[27]</sup> reported that lymph node metastasis and depth of invasion were significantly correlated with 5-year survival in patients aged  $> 70$  years. Pisanu *et al.*<sup>[28]</sup> demonstrated that tumor stage was the only prognostic factor influencing survival for patients aged  $\geq 75$  years. Our results were consistent with these reports and strongly showed that depth of invasion, lymph node metastasis and M1 disease were independent prognostic factors for elderly patients. R0 resection represents the only treatment modality offering possible long-term survival. Positive surgical margin status has been reported to be associated with poor prognosis in patients with gastric cancer<sup>[29,30]</sup>. Our results showed that surgical margin was an independent prognostic factor for all the patients including elderly ones. To ensure a negative surgical margin

**Table 4** Survival analysis of gastric cancer patients aged  $\geq 70$  years

Characteristics	n (%)	5-yr OS	MST (mo)	Univariate analysis		Multivariate analysis	
				$\chi^2$	P (log-rank)	Hazard ratio (95%CI)	P value
Gender				4.009	0.045		
Female	58 (21.2)	29.30%	31			1 (ref)	
Male	215 (78.8)	20.00%	18			1.433 (1.013, 2.029)	0.042
Age (yr)				0.092	0.762		
< 75	164 (60.1)	23.20%	20				
$\geq 75$	109 (39.9)	20.20%	19				
Tumor location				6.055	0.109		
Lower 1/3	78 (28.6)	25.60%	22				
Middle 1/3	58 (21.2)	22.40%	15				
Upper 1/3	106 (38.8)	21.70%	22				
2/3 or more	31 (11.4)	12.90%	16				
Tumor size				8.715	0.003		
< 5 cm	87 (31.9)	32.20%	31			1 (ref)	
$\geq 5$ cm	186 (68.1)	17.20%	17			1.119 (0.816, 1.533)	0.485
Borrmann type				3.221	0.073		
I / II	95 (34.8)	27.40%	26				
III/IV	178 (65.2)	19.10%	18				
Histology				5.610	0.018		
Differentiated	116 (42.5)	28.40%	23			1 (ref)	
Undifferentiated	157 (57.5)	17.20%	18			1.272 (0.956, 1.693)	0.099
Extranodal metastasis				16.966	< 0.001		
Negative	223 (81.7)	24.70%	23			1 (ref)	
Positive	45 (18.3)	10.00%	11			1.248 (0.873, 1.784)	0.225
Surgical margin status				8.957	0.003		
Negative	241 (88.3)	24.10%	22			1 (ref)	
Positive	32 (11.7)	6.30%	12			1.583 (1.048, 2.391)	0.029
Depth of invasion				20.751	< 0.001		
pT <sub>1</sub> -T <sub>3</sub>	45 (16.5)	51.10%	65			1 (ref)	
pT <sub>4</sub>	228 (83.5)	16.20%	17			1.773 (1.118, 2.811)	0.015
Lymph node metastasis				30.397	< 0.001		
pN <sub>0</sub>	87 (31.9)	40.20%	44			1 (ref)	
pT <sub>1</sub> -N <sub>3</sub>	186 (68.1)	13.40%	16			1.658 (1.178, 2.334)	0.004
Distant metastasis				18.941	< 0.001		
M <sub>0</sub>	264 (96.7)	22.70%	21			2.332 (1.143, 4.756)	0.020
M <sub>1</sub>	9 (3.3)	0.00%	6				
Chemotherapy				0.508	0.476		
Yes	36 (13.2)	13.90%	20				
No	237 (86.8)	23.20%	20				
Type of gastrectomy				11.316	0.001		
Subtotal	214 (78.4)	24.80%	22			1 (ref)	
Total	59 (21.6)	11.90%	14			1.305 (0.936, 1.820)	0.117
Extent of lymphadenectomy				1.429	0.232		
D <sub>2</sub>	91 (33.3)	19.80%	17				
D <sub>1</sub>	182 (66.7)	23.10%	22				

Ref: Reference category; OS: Overall survival; MST: Median survival time.

**Table 5** Comparison of survival rate for patients treated with chemotherapy or not stratified by age

Age (yr)	Chemotherapy						$\chi^2$	P value
	Yes			No				
	n	5-yr OS	MST (mo)	n	5-yr OS	MST (mo)		
< 50	71	47.90%	53	95	30.50%	23	8.774	0.003
50-69	142	42.30%	37	339	34.20%	22	7.427	0.006
$\geq 70$	36	13.90%	20	237	23.20%	20	0.508	0.476

OS: Overall survival; MST: Median survival time.

is of paramount importance in gastric cancer surgery.

Extended lymphadenectomy (D<sub>2</sub>) has been reported to yield better survival results in Asian countries, such as Japan and Korea where gastric carcinoma is very

common. Until the 15-year follow-up results of the randomized Dutch D<sub>1</sub>D<sub>2</sub> trial were published, D<sub>2</sub> was not recommended in western countries. The follow-up data showed that OS of patients who had curative resection

was 25% for D1 and 35% for D2 when postoperative deaths (4% in D1 and 10% in D2) were excluded (log-rank  $P = 0.08$ ), however, there were no significant differences in survival between D1 and D2 for patients > 70 years of age (3% for D1 and 13% for D2,  $P = 0.36$ )<sup>[31]</sup>. Also the 5-year follow-up results from the Dutch Gastric Cancer Trial showed no significant survival benefit in the D2 group (47% for D2 and 45% for D1)<sup>[32]</sup>. In elderly patients, surgeons are usually reluctant to perform D2 resection to avoid major complications in the postoperative period<sup>[18,33]</sup>. It was actually reported that none of the elderly patients had lymph node recurrence following limited lymph-node resection<sup>[21]</sup>. In our study, there were no significant differences in OS between D1 and D2 resection for patients aged  $\geq 70$  years (5-year OS: 23.1% for D1 and 19.8% for D2,  $P = 0.232$ ), although those aged < 70 years may benefit from D2 resection. The average life expectancy of elderly patients is short, which may obscure the value of D2 resection, and explain why it is of little benefit in elderly patients. Considering this rather short life expectancy and the increased risk for D2 resection in elderly patients, D1 may be an adequate procedure for elderly patients with gastric cancer.

Many studies have affirmed the survival benefit with postoperative chemotherapy or chemoradiotherapy for gastric cancer<sup>[34-36]</sup>. However, no clinical trial has demonstrated that elderly patients can benefit from postoperative chemotherapy. Although the Adjuvant Chemotherapy of TS-1 for Gastric Cancer (ACTS GC) trial in Japan showed that 3-year survival rates were 80.1% and 70.1% for patients treated with S-1 or surgery alone, respectively, the results concerning elderly patients was not statistically significant<sup>[36]</sup>. FOLFOX-6 has been widely used in gastric cancer patients and has equal efficacy to the XELOX regimen (capecitabine/oxaliplatin), which improved 3-year disease-free survival in the CLASSIC (Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy) trial<sup>[37]</sup>. In our study, improved survival with chemotherapy was only observed in the younger and middle-aged patients, and elderly patients benefited little from chemotherapy. Only 36 (13.2%) elderly patients received postoperative chemotherapy in the present study, therefore, we could not draw any definitive conclusions. A multicenter, larger study is recommended for future investigations.

In conclusion, patients aged  $\geq 70$  years had distinctive characteristics such as male predominance, larger tumor size, more histological differentiated type, higher rate of tumors located in the upper third of the stomach, and advanced TNM stage, but less distant metastasis compared to younger and middle-aged patients. Although there was no significant difference in the prognosis specific to gastric cancer, the elderly patients demonstrated poorer prognosis than the younger and middle-aged patients. Age  $\geq 70$  years was an independent prognostic factor for patients with gastric cancer after gastrectomy. Considering short life expectancy, limited lymph node dissection (D1 resection) is appropriate and postoperative

chemotherapy is possibly unnecessary for elderly patients.

## COMMENTS

### Background

The population of China is growing both larger and older. With the aging of the population, the number of patients aged  $\geq 70$  years with gastric cancer is also increasing. However, it is still unclear whether elderly patients benefit from adjuvant chemotherapy or extended lymph-node dissection.

### Research frontiers

Gastrectomy with D2 lymph node dissection has been increasingly regarded as the standard surgical procedure for most patients with operable gastric cancer, and postoperative adjuvant chemotherapy can improve overall survival. However, it is still controversial whether these therapeutic strategies are suitable for elderly patients. In the present study, authors demonstrated that age  $\geq 70$  years is an independent prognostic factor for patients with gastric cancer after gastrectomy, D1 resection is appropriate, and postoperative chemotherapy is possibly unnecessary for elderly patients.

### Innovations and breakthroughs

Few studies have compared the characteristics and prognosis of gastric cancer among younger, middle-age and elderly patients. In this study, authors compared characteristics and prognosis among the three age groups and found that elderly patients had distinctive characteristics such as male predominance, larger tumor size, more histological differentiated type, higher rate of tumors located in the upper third of the stomach, and advanced tumor-node-metastasis stage, but less distant metastasis compared to younger and middle-aged patients. Furthermore, cancer-specific survival was almost equal between the elderly and the younger patients, but overall mortality was higher in the former group.

### Applications

By understanding the characteristics and prognostic factors of elderly gastric cancer patients, this study may provide a reference for treatment planning for elderly patients with gastric cancer in China.

### Terminology

Extranodal metastasis was defined as the presence of tumor cells in extramural soft tissue that was discontinuous with either the primary lesion or locoregional lymph nodes.

### Peer review

The authors reported the characteristics and prognosis of gastric cancer in 920 patients who were treated in the authors' hospital. The manuscript has been well designed and conducted. It revealed that for the elderly patient subset, in comparison with younger patient subsets, distant metastasis was less frequent, the efficacy of adjuvant chemotherapy was less effective, and the benefit of extended lymph-node dissection was unclear. A cancer-specific death rate was almost equal between the elderly subset and the younger subset, but overall mortality was higher in the former patient group. These data are very informative for the planning of strategy to treat the elderly patients with gastric cancer in China. The idea of the manuscript has potential instruction for clinicians.

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## Computed tomography findings of pneumatosis and portomesenteric venous gas in acute bowel ischemia

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### Abstract

**AIM:** To use more representative sample size to evaluate whether computed tomography (CT) scan evidence of the concomitant presence of pneumatosis and portomesenteric venous gas is a predictor of transmural bowel necrosis.

**METHODS:** Data from 208 patients who were referred for a diagnosis of bowel ischemia were retrospectively reviewed. Only patients who underwent a surgical intervention following a diagnosis of bowel ischemia who also had a post-operative histological confirmation of such a diagnosis were included. Patients were split into two groups according to the presence of histological evidence of transmural bowel ischemia (case group) or partial bowel ischemia (control group). CT images were reviewed for findings of ischemia, including mural thickening, pneumatosis, bowel distension, portomesenteric venous gas and arterial or venous thrombi.

**RESULTS:** A total of 248 subjects who underwent surgery for bowel ischemia were identified. Among the 208 subjects enrolled in our study, transmural bowel necrosis was identified in 121 subjects (case group), and partial bowel necrosis was identified in 87 subjects (control group). Based on CT findings, including mural thickening, bowel distension, pneumatosis, pneumatosis plus portomesenteric venous gas and presence of thrombi or emboli, there were no significant differences between the case and control groups. The concomitant presence of pneumatosis and porto-mesenteric venous gas showed an odds ratio of 1.95 (95%CI: 0.491-7.775,  $P = 0.342$ ) for the presence of transmural necrosis. The presence of pneumatosis plus porto-mesenteric venous gas exhibited good specificity (83%) but low sensitivity (17%) in the identification of transmural bowel infarction. Accordingly, the positive and negative predictive values were 60% and 17%, respectively.

**CONCLUSION:** Although pneumatosis plus porto-mesenteric venous gas is associated with bowel ischemia, we have demonstrated that their co-occurrence cannot be used as diagnostic signs of transmural necrosis.

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**Key words:** Bowel ischemia; Pneumatosis; Mesenteric venous gas; Computed tomography

**Core tip:** Although the finding of pneumatosis plus porto-mesenteric venous gas is useful in verifying transmural necrosis with a specificity of 83%, the very low sensitivity of 17% indicates that the diagnosis of transmural necrosis cannot be excluded based on a normal findings. Thus, our results appear to be encouraging by indicating that neither portomesenteric venous gas nor pneumatosis were pathognomonic of bowel transmural infarction.

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## INTRODUCTION

Acute bowel ischemia is an urgent, life-threatening vascular condition with a mortality rate of approximately 70%<sup>[1-3]</sup>.

The slightest suspicion of mesenteric ischemia should be ruled out through imaging diagnostics<sup>[1]</sup>. In such a case, the diagnostic tool of choice is computed tomography (CT) angiography using an early arterial contrast medium bolus injection, followed by an evaluation of the primary axial layers and a multiplanar reconstruction of the imaging technique<sup>[1]</sup>.

In recent years, CT has increasingly been used to evaluate patients with clinical signs and symptoms of ischemic bowel disease. Depending on the severity and the extent of disease, intestinal ischemia may be detectable by CT based on a spectrum of findings, including intestinal pneumatosis, bowel wall thickening, portomesenteric venous gas and arterial or venous thrombi<sup>[4-7]</sup>.

Pneumatosis plus portomesenteric venous gas have been considered signs of advanced disease that usually indicate irreversible injury and transmural necrosis<sup>[6-8]</sup>.

Although Wiesner *et al*<sup>[9]</sup> and Kernagis *et al*<sup>[6]</sup> found an association between transmural bowel infarction and the comorbid presence of pneumatosis and porto-mesenteric venous gas, these studies represented only reports and included up to seven patients.

The purpose of our study was to use a more representative sample size to evaluate whether CT scan evidence of the concomitant presence of pneumatosis and portomesenteric venous gas is a predictor of transmural bowel necrosis.

## MATERIALS AND METHODS

Data of patients referred during a 13-year period (from January 2000 to December 2012) to the University of Naples "Federico II" and to the "Fatebenefratelli" Hospital of Naples with diagnoses of bowel ischemia were retrospectively reviewed. Only patients who underwent a surgical intervention following a diagnosis of bowel ischemia and obtained a post-operative histological confirmation of the diagnosis were included.

The patients were split into two groups according to the presence of histological evidence of transmural bowel ischemia (case group) or partial bowel ischemia (control group). Medical records were reviewed to determine the demographic and clinical characteristics of the patients in both groups.

CT images were reviewed for findings that were con-

sistent with the most common signs of ischemia that had been identified in previous literature<sup>[4-9]</sup>, including mural thickening, pneumatosis, bowel distension, portomesenteric venous gas and arterial or venous thrombi.

A measurement of 2-3 mm as the upper limit of normal thickness has been used<sup>[10]</sup>, and air bubbles or continuous bands of air in the bowel wall were considered to be a sign of pneumatosis<sup>[9]</sup>. Gas in the mesenteric veins or gas in the intrahepatic branches of the portal vein were indicative of gas in the portomesenteric circulation (Figure 1)<sup>[9]</sup>, and only the emboli or thrombi in the mesenteric arteries and veins that were clearly shown on contrast-enhanced CT images were assessed<sup>[11]</sup>.

To minimise selection bias, all CT scans were analysed by an observer with 30 years of experience related to the interpretation of CT who was blinded to the surgical and pathological findings and to the eventual clinical outcomes for each patient included in the chart review.

Patients without a contrast-enhanced CT obtained with 150 mL of iodinate contrast media and 5- and 7-mm slice collimations (pitch, 1.3:1, 200-220 mAs), reconstructed with a soft-tissue algorithm, were excluded from the study.

Superficial damage of the bowel mucosa and submucosa was classified as partial bowel ischemia before the development of transmural infarction based on the histological examination<sup>[12]</sup>.

The results were then correlated with the clinical and pathological data to determine the frequency with which pneumatosis plus portomesenteric venous gas was associated with irreversible transmural infarction.

## Statistical analysis

Statistical analysis was performed with SPSS 16.0. The Yates corrected  $\chi^2$  test was used to evaluate differences in categorical variables, and the independent samples *t* test was used to analyse continuous variables. To adjust for covariates and to make predictions, linear and logistic regression models were used. Statistical significance was accepted when the *P* value was less than 0.05.

## RESULTS

A total of 248 subjects who underwent surgery for bowel ischemia were identified. Of these, 208 subjects were included in the analysis; 28 subjects were excluded because they lacked adequate CT images, and 12 subjects were excluded for lacking a properly conducted pathological differentiation of mural bowel necrosis.

Among the 208 subjects enrolled in our study, transmural bowel necrosis was identified in 121 subjects (case group), and partial bowel necrosis was identified in 87 subjects (control group).

There were no significant demographic, clinical and laboratory differences between the case and control groups (Table 1). Based on a group comparison of CT findings of mural thickening, bowel distension, pneumatosis, pneumatosis plus portomesenteric venous gas



Figure 1 A case of intra-hepatic venous gas without trans-mural necrosis.

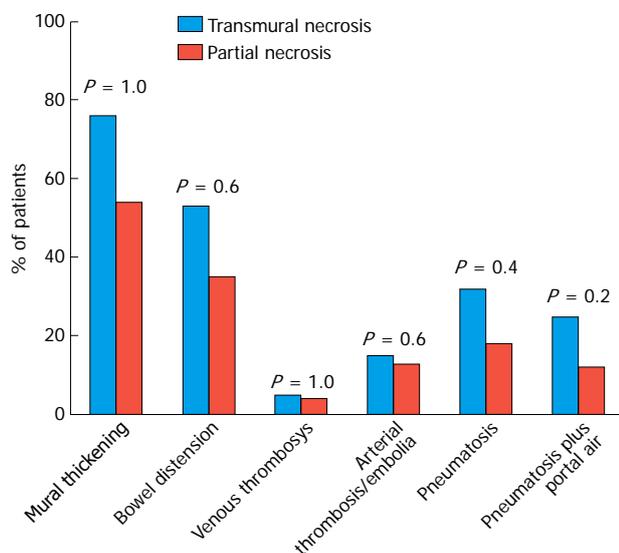


Figure 2 Association between the evaluated computed tomography findings and the presence of transmural or partial necrosis.

and the presence of thrombi or emboli, no significant differences were observed between the case and control groups (Figure 2).

A multivariate analysis indicated that after adjusting for major clinical and demographic patient characteristics, none of the CT findings were found to be predictive of transmural necrosis. Specifically, the concomitant presence of pneumatosis and porto-mesenteric venous gas showed an OR of 1.95 (95%CI: 0.491-7.775,  $P = 0.342$ ) for the presence of transmural necrosis. Similarly, the presence of isolated pneumatosis ( $P = 0.840$ ), of mural thickening ( $P = 0.974$ ) and of thrombi ( $P = 0.483$ ) did not predict the presence of transmural necrosis.

The presence of pneumatosis plus porto-mesenteric venous gas showed good specificity (83%) but low sensitivity (17%) in the identification of transmural bowel infarction. Accordingly, the positive and negative predictive values were 60% and 17%, respectively. Specifically, we registered 96 cases of transmural necrosis without pneumatosis plus porto-mesenteric venous gas (false negatives) and 12 cases of transmural necrosis with

Table 1 Demographic and clinical characteristics of the included patients in both groups  $n$  (%)

Characteristics	Transmural necrosis group	Partial necrosis group	$P$ value
Age (mean $\pm$ SD), yr	66.6 $\pm$ 8.1	66.2 $\pm$ 5.9	0.7
Sex (male)	61 (50.8)	43 (49.4)	1.0
Hypertension	57 (47.1)	43 (49.4)	0.7
Type 2 diabetes	26 (21.5)	20 (23.0)	0.8
Chronic pulmonary disease	21 (17.3)	13 (14.9)	0.7
Atrial fibrillation	10 (8.3)	14 (16.0)	0.1
Chronic renal failure	7 (5.8)	6 (6.9)	0.7
Coronary artery diseases	15 (12.4)	15 (17.2)	0.4
Pain	121 (100.0)	87 (100.0)	1.0
White blood cell count > 15000/mL	75 (62.0)	56 (64.4)	0.7
pH < 7.2 on blood gas analysis	56 (46.3)	36 (41.4)	0.5
Lactate in serum > 4 mmol/L	69 (57.0)	55 (63.2)	0.3

pneumatosis plus porto-mesenteric venous gas (false positives). In a ROC curve analysis, the evidence of pneumatosis plus porto-mesenteric venous gas was associated with an area under the curve of 0.50 (95%CI: 0.420-0.580) for predicting the presence of transmural bowel infarction.

## DISCUSSION

To our knowledge, no previous group has evaluated a large series of patients to statistically analyse whether the presence of pneumatosis intestinalis and portomesenteric venous gas facilitates the prediction of ischemic bowel wall damage severity.

Acute intestinal ischemia is pathogenetically and histologically divided into four primary clinical categories, including acute mesenteric embolus, acute mesenteric thrombus, non-occlusive disease and mesenteric vein thrombosis<sup>[3,13-15]</sup>.

Although acute intestinal ischemia is generally uncommon, as it accounts for < 1 in 1000 hospital admissions<sup>[16,17]</sup>, it is a highly complex clinical problem faced during the daily routines of surgeons, gastroenterologists, and radiologists<sup>[4]</sup> due to the difficulty of providing a diagnosis.

The severity of bowel ischemia may range from mild and transient superficial damage of the bowel mucosa to life-threatening transmural bowel infarction<sup>[18,19]</sup>.

Early diagnosis and treatment determine the overall outcome. As a result, increased awareness among physicians who have clinical patients with suspected mesenteric ischemia and a timely diagnostic workup and initiation of therapy are the keys to saving a patient's life<sup>[4,20]</sup>.

However, the absence of specific symptoms upon clinical examination may make an appropriate assessment more difficult<sup>[4,21]</sup>. Typically, the course of an ischemic condition is triphasic. During the initial stage (0-6 h), there is intense, acute abdominal pain that is often accompanied by shock and diarrhoea. These signs are normally followed by a phase with little evidence of symptoms, known as the silent phase (7-12 h), which is

characterised by dull abdominal pain, intestinal paralysis, and a rapid deterioration of the general condition. During the final phase (12–24 h), manifest ileus and bacterial peritonitis with sepsis are evident, and multi-organ failure has begun. Satisfactory treatment results are only possible in the early stage (0–12 h)<sup>[1]</sup>.

Necrosis is typically accompanied by increased serum lactate (> 4 mmol/L), increased CRP (> 10 mg/L), and leukocytosis (> 15000/mL). Furthermore, the blood gas analysis indicates an ischemic necrosis exhibiting acidosis with a pH of < 7.2 and a base excess of minus 7–8 mmol/L<sup>[1]</sup>. Despite these signs, no laboratory-chemical parameters currently exist that are specifically designed to confirm intestinal necrosis.

Currently, contrast-enhanced biphasic multidetector row helical CT of the abdomen is conducted as the primary imaging modality in many medical centres. This technique enables the imaging of the entire abdomen with high temporal and spatial resolution in the arterial and portal venous phases<sup>[4]</sup>.

In addition to high sensitivity and specificity, multidetector CT provides the following advantages: it is a rapid and non-invasive technique that is available 24 h a day in most acute-care medical centres. Due to its high sensitivity, specificity, availability, and non-invasiveness, the multidetector CT is a suitable modality for the diagnosis of acute mesenteric ischemia. Furthermore, it is extremely helpful for triage patients who require subsequent conventional angiography, surgery, or clinical surveillance<sup>[4]</sup>.

Aschoff *et al.*<sup>[5]</sup> reported a sensitivity of 93% and a specificity of 100% of CT angiography for the diagnosis of acute mesenteric ischemia. Diagnosis was based on an analysis of both vascular occlusions and the consequences of tissue damage, such as intestinal pneumatosis, bowel wall thickening, portomesenteric venous gas, or solid organ infarction<sup>[4]</sup>.

An association between pneumatosis intestinalis and portomesenteric venous gas is a strong indicator of the presence of mesenteric infarction or segmental ischemia, and it is therefore an indication for emergency exploratory surgery<sup>[9,22–26]</sup>.

Data from the literature suggest that the combination of pneumatosis intestinalis and portal venous gas is associated with the presence of bowel ischemia in approximately 70% of all cases<sup>[22,27,28]</sup>. We extended these results by evaluating the association of this CT finding with the severity of mural necrosis.

Bowel pneumatosis is an imaging phenomenon that can represent the presence of gas in the bowel wall<sup>[22]</sup>. Hepatic portal venous gas is defined radiologically as tubular areas of decreased attenuation in the liver periphery<sup>[29,30]</sup>.

Although the etiology of pneumatosis appears to be multifactorial, the exact causes are not known. Two primary theories have been proposed in the medical literature. A mechanical theory hypothesises that gas dissects into the bowel wall from either the intestinal lumen or the lungs, *via* the mediastinum, due to some mechanism

that causes increased pressure (*i.e.*, bowel obstruction or emphysema). In contrast, a bacterial theory proposes that gas-forming bacilli enter the submucosa through mucosal rents or increased mucosal permeability, thereby producing gas within the bowel wall. A combination of both theories is also plausible. Bacterial overgrowth in the gastrointestinal tract arising from a variety of causes can lead to excessive hydrogen gas production, bowel distension, and subsequently, the dissection of intraluminal hydrogen gas into the bowel wall<sup>[25]</sup>. In the case of ischemia, pneumatosis has been considered to be an ominous radiographic finding, particularly if it is associated with portomesenteric venous gas. In some articles, pneumatosis has been described as an advanced sign of ischemic bowel disease, usually indicating irreversible injury and transmural necrosis<sup>[6–8]</sup>.

Wiesner *et al.*<sup>[9]</sup> supports the hypothesis that patients with pneumatosis and portomesenteric venous gas are more likely to exhibit transmural infarction. Similarly, Kernagis *et al.*<sup>[6]</sup> determined that patients with CT findings of pneumatosis and portomesenteric venous gas were more likely to have transmural infarction than those with pneumatosis alone.

However, these studies share several limitations, as they were retrospective investigations with small sample sizes (up to seven patients), which may have magnified the effects of selection bias on the study population.

Although pneumatosis plus porto-mesenteric venous gas is associated with bowel ischemia, we have demonstrated that it cannot be used as a diagnostic sign of transmural necrosis. No statistical associations were identified between transmural infarction and the presence of pneumatosis plus porto-mesenteric venous gas, according to both univariate and multivariate analyses. Although the finding of concomitant pneumatosis plus porto-mesenteric venous gas is useful in verifying transmural necrosis with a specificity of 83%, the very low sensitivity of 17% indicates that the diagnosis of transmural necrosis cannot be excluded based on normal findings. Some limitations of our study warrant discussion. A major concern is the retrospective design of the present study, which has inherent limitations and biases. Moreover, we included only patients who underwent surgical intervention in the analyses. As a result, we are likely to have selected patients with more severe clinical presentations. Thus, further ad hoc-designed prospective studies with adequate sample sizes are needed to evaluate whether pneumatosis intestinalis and portomesenteric venous gas facilitates the prediction of ischemic bowel wall damage severity. However, our encouraging results appear to suggest that neither portomesenteric venous gas nor pneumatosis were pathognomonic of bowel trans-mural infarction.

## COMMENTS

### Background

Pneumatosis plus portomesenteric venous gas have been considered signs of

advanced disease, usually indicating irreversible injury and transmural necrosis. Although some authors have identified an association between transmural bowel infarction and the presence of concomitant pneumatosis plus portomesenteric venous gas, these studies only represented reports that included up to seven patients.

### Research frontiers

Although a strong indicator of the presence of mesenteric infarction or segmental ischemia is the association of pneumatosis intestinalis with portomesenteric venous gas, their study demonstrates that these findings are not useful in assessing the presence of transmural necrosis.

### Innovations and breakthroughs

The finding that pneumatosis plus porto-mesenteric venous gas is useful in verifying transmural necrosis with a specificity of 83%, but its very low sensitivity of 17% indicates that the diagnosis of transmural necrosis cannot be excluded based on normal findings.

### Applications

Their study emphasises that pneumatosis and portomesenteric venous gas cannot be considered signs of transmural necrosis.

### Terminology

A superficial damage of bowel mucosa and submucosa was histologically assessed as partial bowel ischemia before the development of transmural infarction. Air bubbles or continuous bands of air in the bowel walls were considered to be signs of pneumatosis, whereas gas in the mesenteric veins or gas in the intrahepatic branches of the portal vein were indicative of gas in the portomesenteric circulation at the time of computed tomography (CT) examination.

### Peer review

This is an interesting study in which authors use more representative sample size to evaluate whether CT scan evidence of the concomitant presence of pneumatosis and portomesenteric venous gas is a predictor of transmural bowel necrosis. The results are interesting and suggest that although pneumatosis plus porto-mesenteric venous gas is associated with bowel ischemia, they have demonstrated that their co-occurrence cannot be used as diagnostic signs of transmural necrosis.

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## Prevalence of *Helicobacter pylori* virulence genotypes among children in Eastern Turkey

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common *vacA* subtype was s1a (79.6%). However, the s2 gene was found less frequently with an isolation rate of 8.2% of the *H. pylori* isolates. The genotypes *iceA2* and *vacA s1m2* were the most frequently found types in children with antral nodularity. In addition, the genotypes *iceA1*, *babA2* and *vacA s1m1* were found in similar ratios in all the *H. pylori* isolates obtained from children with peptic ulcer. The genotypes *vacA s2m1* and s1c were not observed in any of isolates studied.

**CONCLUSION:** This study showed that *vacA s1m2*, *cagA* and *iceA2* were the most common genotypes, and no association between antral nodularity and genotypes was observed.

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**Key words:** *Helicobacter pylori*; Children; Genotype; Polymerase chain reaction

### Abstract

**AIM:** To identify the virulence genotypes of *Helicobacter pylori* (*H. pylori*) if present in children in Eastern Turkey and if those genotypes are mostly associated with severe clinical presentations.

**METHODS:** A total of 49 *H. pylori* positive Turkish children (42 with antral nodularity and 7 with peptic ulcer) who underwent upper gastrointestinal endoscopy with abdominal symptoms during the period from March 2011 to September 2012 were enrolled in this study. Antral nodularity was diagnosed endoscopically by two of the authors. We determined for the presence of *cagA*, *vacA*, *cagE*, *iceA* and *babA2* genotypes of *H. pylori* isolates in DNA obtained directly from frozen gastric biopsy samples by polymerase chain reaction test using specific primers.

**RESULTS:** Of the 49 *H. pylori* isolates studied, 61.2%, 91.8%, 22.4%, 28.6%, 57.1% and 40.8% were positive for the *cagA*, *vacA* s1, *cagE*, *iceA1*, *iceA2* and *babA2* genes, respectively. We showed that the most

**Core tip:** In this research we have attempted to determine the prevalence of some genotypes of *Helicobacter pylori* (*H. pylori*) among children in Eastern Turkey and to investigate the relationship between these genotypes with antral nodularity. Identifying the virulence genes among *H. pylori* isolates in children would allow for the development of new treatments and eradication policies in adults. The study results suggest that there was no significant association between antral nodularity and the presence of genotypes ( $P > 0.05$ ).

Ozbey G, Dogan Y, Demiroren K. Prevalence of *Helicobacter pylori* virulence genotypes among children in Eastern Turkey. *World J Gastroenterol* 2013; 19(39): 6585-6589 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6585.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6585>

### INTRODUCTION

*Helicobacter pylori* (*H. pylori*) infection is generally acquired

during childhood, persists throughout life unless treated with antibiotics, and the infection is usually associated with the development of several gastroduodenal diseases such as gastritis, peptic ulcer, gastric carcinoma and mucosa-associated lymphoid tissue lymphoma<sup>[1-3]</sup>.

The cytotoxin associated gene A (*cagA*) being a marker for the presence of the *cag* pathogenicity island (*cag* PAI) was the first recognized virulence gene in the *H. pylori* genome in both adults and children<sup>[3,4]</sup>. Cytotoxin associated gene E (*cagE*) is also a member of the *cag* PAI, and has been described as a potential virulence factor associated with duodenal ulcer in children<sup>[5]</sup>. The vacuolating cytotoxin (*vacA*) gene exists in different subtypes, varying in the signal (s1 or s2) and middle (m1 or m2) regions<sup>[6,7]</sup>. *H. pylori vacA* alleles differ in their ability to express an active toxin<sup>[7]</sup>. Inducement occurs *via* contact with the epithelium gene (*iceA*), has two allelic variants (*iceA1* and *iceA2*), and has been determined through its upregulation after adherence of *H. pylori* to the gastric epithelium<sup>[8,9]</sup>. The blood adhesion binding antigen A (*babA*) adhesion of *H. pylori*, encoded by the *babA2* gene is an outer membrane protein that binds to the fucosylated histo-blood group antigens on the surface of gastric epithelial cells<sup>[10]</sup>.

Despite a high prevalence of *H. pylori* infection among children and adults in Turkey, the published data on geographic distribution of the virulence genes in *H. pylori* strains among Turkish children are very limited, and relatively few studies have been reported on the prevalence of the *cagA* gene of *H. pylori* in Turkish children<sup>[11-13]</sup>. This study was performed to determine the prevalence of some virulence genes of *H. pylori* which were not previously reported among children in Eastern Turkey, and to investigate the association between these genotypes with clinical disease.

## MATERIALS AND METHODS

A total of 49 *H. pylori* isolates were investigated for the presence of virulence genotypes. These isolates [by polymerase chain reaction (PCR)] were recovered from 101 Turkish children (53 girls and 48 boys, ranging between 4 and 18 years old, average 12 years) who underwent upper gastrointestinal endoscopy with abdominal symptoms at the clinic of the Pediatric Gastroenterology Department at the Firat University Hospital between March 2011 and September 2012. Antral nodularity was defined as being endoscopically characterized by the irregular appearance of the mucosa as like that of a "cobblestone pavement"<sup>[14]</sup>. Also, the presence of ulcers was determined by endoscopic examination.

Our study was approved by the Medical Ethics Committee of Firat University. All patients received informed consent that was signed by their parents before endoscopic procedures.

### Isolation of *H. pylori* DNA and PCR detection of its genotypes

*H. pylori* DNA was prepared using the QIAamp DNA

mini kit (Qiagen, Germany) following the manufacturer's instructions. The extracted DNA was kept at -20 °C until tested.

PCR was carried out using oligonucleotide primers targeting the 298 bp fragment of the *cagA* gene; the fragment 259 bp or 286 bp in size for type s1 or s2; the 190, 187 and 213 bp fragments for s1a, s1b, and s1c; the 567 bp and 642 bp fragments for m1 and m2; the 508 bp fragment of the *cagE* gene; the 247-bp fragment of *iceA1*; the 229 or 334-bp fragments of *iceA2*; and the 271 bp fragment of the *babA2* gene, in order to amplify the *cagA*, *vacA*, *cagE*, *iceA* and *babA2* genes of the *H. pylori* isolates<sup>[7,15-20]</sup>. Ten µL of each PCR product was subjected to electrophoresis in a 1.5% (w/v) agarose gel.

All reactions were performed with positive controls containing the DNA of the HP 26695, HP J99, and some clinical isolates supplied by Dr. Yoshio Yamaoka, along with negative controls containing all PCR components with distilled water to substitute the DNA sample.

### Statistical analysis

Statistical analysis was performed by statistical software program SPSS for Windows version 12.00 (SPSS, Chicago, IL, United States). The correlation between *H. pylori* genotypes and antral nodularity was assessed by Fischer's exact and  $\chi^2$  tests. A *P*-value of less than 0.05 was considered statistically significant.

## RESULTS

Table 1 summarizes the prevalence of *cagA*, *vacA*, *cagE*, *iceA* and *babA2* genes with antral nodularity and peptic ulcers. The number of children with peptic ulcers in the present study was low; therefore further analysis was not carried out.

The *cagA* gene was found in 30 of the 49 isolates (61.2%). In our study, the *vacA* genes were observed in all isolates. The most predominant subtype was s1a (79.6%), followed by s1b (12.2%), then s2 (8.2%). The genotype s1m2, which was predominant in this study, was observed in 28 (57.1%) isolates. However, the genotypes s1m1 and s2m2 were detected in 17 (34.7%) and 4 (8.2%) isolates, respectively. Furthermore, the genotype *vacA* s2m1 and subtype s1c were not found in any of the isolates. The prevalence of *cagE* gene in children with antral nodularity and peptic ulcer was 8 out of 42 (19%) and 3 out of 7 (42.9%) isolates, respectively. The *iceA* gene was not observed in 4 of the 49 isolates. The *iceA2* gene was positive in 28 (57.1%) isolates, while *iceA1* was detected in 14 (28.6%) isolates. Three isolates (6.1%) were positive for both *iceA1* and *iceA2*. The prevalence of *iceA1* was higher in patients with peptic ulcers (57.1%), with no significance difference observed compared to patients with antral nodularity (23.8%). The *babA2* gene was detected in 20 (40.8%) samples. The *babA2* showed a higher proportion (57.1%) in patients with peptic ulcer compared to patients with antral nodularity (38.1%).

We emphasized no significant association between antral nodularity and the presence of the genotypes (*P* > 0.05).

**Table 1** The prevalence of the virulence factor genes of *Helicobacter pylori* from children with antral nodularity and peptic ulcer *n* (%)

Virulence factor genes	Antral nodularity ( <i>n</i> = 42)	Peptic ulcer ( <i>n</i> = 7)	Total ( <i>n</i> = 49)
<i>cagA</i>	25 (59.5)	5 (71.4)	30 (61.2)
<i>vacA</i> s1	38 (90.5)	7 (100)	45 (91.8)
<i>vacA</i> s1a	32 (76.2)	7 (100)	39 (79.6)
<i>vacA</i> s1b	6 (14.3)	0 (0)	6 (12.2)
<i>vacA</i> s2	4 (9.5)	0 (0)	4 (8.2)
<i>vacA</i> m1	13 (31)	4 (57.1)	17 (34.7)
<i>vacA</i> m2	29 (69)	3 (42.9)	32 (65.3)
<i>vacA</i> s1/m1	13 (31)	4 (57.1)	17 (34.7)
<i>vacA</i> s1/m2	25 (59.5)	3 (42.9)	28 (57.1)
<i>vacA</i> s2/m2	4 (9.5)	0 (0)	4 (8.2)
<i>cagE</i>	8 (19)	3 (42.9)	11 (22.4)
<i>iceA1</i>	10 (23.8)	4 (57.1)	14 (28.6)
<i>iceA2</i>	25 (59.5)	3 (42.9)	28 (57.1)
Both <i>iceA1</i> and <i>iceA2</i>	3 (7.1)	0 (0)	3 (6.1)
Non <i>iceA1</i> and <i>iceA2</i>	4 (9.5)	0 (0)	4 (8.2)
<i>babA2</i>	16 (38.1)	4 (57.1)	20 (40.8)

## DISCUSSION

Although only one study on virulence genes of *H. pylori* has been performed in adults in the Elazig Province in Eastern Turkey<sup>[21]</sup>, there is no data related to the prevalence of *H. pylori* genotypes among children in this region. However, there are a few studies on determining the prevalence of the *cagA* gene of *H. pylori* in Turkish children<sup>[11-13]</sup>.

The prevalence of the *cagA* gene in children among European countries varies from 22.4% to 76%<sup>[2,22]</sup>. Earlier studies performed in Turkish children showed the prevalence of the *cagA* gene was 55%-74.4%<sup>[11-13]</sup>. In this study, we detected the prevalence of 61.2% of the *cagA* gene among Turkish children. The inconsistent findings may be due to adaptation of *H. pylori* to the environment in different geographic regions<sup>[23]</sup>. Some studies had confirmed a significant correlation between the severity of histological changes and the presence of the *cagA* gene in the *H. pylori* genome<sup>[23-26]</sup>, whereas others<sup>[11,27-29]</sup> have not emphasized this association.

It has been demonstrated that the geographic distribution for *vacA* alleles differs in many countries around the world<sup>[30]</sup>; *s1c* is the common strain in East Asia, while *s1a* is the prevalent strain in Northern Europe, and *s1b* in Portugal and Spain<sup>[19]</sup>. The majority of *H. pylori* isolates identified as *s1a*; however, no subtypes *s1c* and *s2* were found in this study. The *vacA* *s1m1*, *s1m2*, and *s2m2* genotypes were found in 34.7%, 57.1%, and 8.2%, respectively. No *s2m1* genotype was detected in the present study. Our data is consistent with the results reported in Poland<sup>[31]</sup> and Shanghai<sup>[32]</sup> where the *s1m2* was the most prevalent genotype. In contrast, other predominant *vacA* genotypes were reported in Brazil, Slovenia, the Midwestern United States (*s1m1*), and Spain (*s2m2*)<sup>[25,28,33,34]</sup>.

The prevalence of the *iceA1* genotype was found

to be 14% in Brazil<sup>[24]</sup>, 37% in Israel<sup>[28]</sup>, 44% in North America<sup>[27]</sup>, and 62% in Slovenia<sup>[23]</sup>. The prevalence (28.6%) of the *iceA1* gene in this study was similar to the Brazilian population (14%)<sup>[24]</sup>, but lower than in Korea (76%)<sup>[35]</sup>. Although it has been shown that the *iceA1* gene is associated with ulcer disease in adults<sup>[19]</sup>, no significant association between the *iceA1* subtype and disease severity was found which is concordant with other studies<sup>[23,27,28]</sup>. We found that the *iceA2* gene (57.1%) was the predominant genotype, supporting the findings of pediatric studies in Brazil (68.9%), Israel (52%), and the Midwestern United States (84%)<sup>[25,28,33]</sup>.

The prevalence of *cagE* was found in 24.5% of *H. pylori* isolates in Israel<sup>[28]</sup>, 59% in Canada<sup>[5]</sup>, and 41.7% in Bulgaria<sup>[26]</sup>. The *cagE* gene was detected in 11 (22.4%) out of 49 isolates, and no significant association was found between the *cagE* and peptic ulcers in children in this study, consistent with a study by Benenson *et al.*<sup>[5]</sup>. However, another study showed just such an association<sup>[5]</sup>. Furthermore, we observed that the *cagE* gene was predominantly detected in *H. pylori* isolates from children with peptic ulcers. Because of the relatively low number of children with peptic ulcers, statistical analysis was not carried out.

The prevalence of *H. pylori* *babA2* was 17.2% in Portugal, 36% in the Midwestern United States, 84.4% in Brazil, and 66.7% in Bulgaria<sup>[2,25,26,33]</sup>. In the present study, the *babA2* gene was detected in 40.8% of the *H. pylori* isolates. The low prevalence of *babA2* in children can also be explained by the fact that *H. pylori* strains exhibit different patterns of adherent to gastric mucosa cells in adults and children, pointing out the importance of host characteristics in the selection of determinants of the infecting strain<sup>[36,37]</sup>.

In conclusion, we feel that the clinical presentations observed are not correlated with the presence of the virulence genotypes because of small numbers of *H. pylori* isolates. However, the identification of virulence genotypes in this study will be important for future policies for the eradication of *H. pylori* in order to prevent severe diseases in adults.

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## COMMENTS

**Background**

There are a few studies on the virulence genes of *Helicobacter pylori* (*H. pylori*) in Turkish children and the correlation of these genotypes with clinical outcome. The present study aimed to detect the prevalence of *cagA*, *vacA*, *cagE*, *iceA* and *babA2* genotypes of *H. pylori* in children in Eastern Turkey and to assess the association between these virulence genotypes and antral nodularity.

**Research frontiers**

In this study, the authors investigated the prevalence of *cagA*, *vacA*, *cagE*, *iceA* and *babA2* genotypes of *H. pylori* among children in Eastern Turkey and evaluated the association between these genotypes with antral nodularity. There was no significant association between virulence factor genes with antral nodularity.

**Innovations and breakthroughs**

This is the first study on the prevalence of the *vacA*, *cagA*, *cagE*, *iceA* and *babA2* genes among children in Eastern Turkey and the correlation of these virulence factor genes with antral nodularity. This research is useful not only in developing future strategies to control and eradicate *H. pylori* infection but also to contribute a better understanding of the epidemiology of *H. pylori* infection. In this study, they examined small numbers of *H. pylori* isolates. More large population and genotyping studies are needed for the development of the future policies to eradicate *H. pylori* infection.

**Applications**

The data obtained from this study will be useful in developing the future policies for the eradication of *H. pylori* in order to prevent severe diseases in adults.

**Peer review**

The authors studied the prevalence of *H. pylori* virulence genotypes among children in Eastern Turkey. This is a useful paper on a topic for which there is, as yet little information. It will certainly contribute to knowledge on the issue.

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## Bariatric surgery and diabetes remission: Sleeve gastrectomy or mini-gastric bypass?

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### Abstract

**AIM:** To investigate the weight loss and glycemic control status [blood glucose, hemoglobin A1c (HbA1c) and hypoglycaemic treatment].

**METHODS:** The primary risk factor for type 2 diabetes is obesity, and 90% of all patients with type 2 diabetes are overweight or obese. Although a remarkable effect of bariatric surgery is the profound and durable resolution of type 2 diabetes clinical manifestations, little is known about the difference among various weight loss surgical procedures on diabetes remission. Data from patients referred during a 3-year period (from January 2009 to December 2011) to the University of Naples "Federico II" diagnosed with obesity and diabetes were retrieved from a prospective database. The patients were split into two groups according to the surgical intervention performed [sleeve gastrectomy (SG) and mini-gastric bypass (MGB)]. Weight loss and glycemic control status (blood glucose, HbA1c and hypoglycaemic treatment) were evaluated.

mic treatment) were evaluated.

**RESULTS:** A total of 53 subjects who underwent sleeve gastrectomy or mini-gastric bypass for obesity and diabetes were screened for the inclusion in this study. Of these, 4 subjects were excluded because of surgical complications, 7 subjects were omitted because young surgeons conducted the operations and 11 subjects were removed because of the lack of follow-up. Thirty-one obese patients were recruited for this study. A total of 15 subjects underwent SG (48.4%), and 16 underwent MGB (51.6%). After adjusting for various clinical and demographic characteristics in a multivariate logistic regression analysis, high hemoglobin A1c was determined to be a negative predictor of diabetes remission at 12 mo (OR = 0.366, 95%CI: 0.152-0.884). Using the same regression model, MGB showed a clear trend toward higher diabetes remission rates relative to SG (OR = 3.780, 95%CI: 0.961-14.872).

**CONCLUSION:** Although our results are encouraging regarding the effectiveness of mini-gastric bypass on diabetes remission, further studies are needed to provide definitive conclusions in selecting the ideal procedure for diabetes remission.

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**Key words:** Bariatric surgery; Sleeve; Bypass; Obesity and diabetes

**Core tip:** Duodenum exclusion could suggest the potential superiority of mini-gastric bypass over sleeve gastrectomy to obtain diabetes remission. This mechanism could suggest the potential superiority of mini-gastric bypass over sleeve gastrectomy to obtain diabetes remission. Thus, although the gold standard for diabetes remission is still the Roux-en-y gastric bypass, being similar mechanisms of diabetes remission involved and being easier to be performed, the mini-gastric bypass

could become a valuable alternative.

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## INTRODUCTION

Severe obesity is one of the major problems in Western Countries and is associated with several comorbidities and disabling diseases (*e.g.*, cardiovascular disease, metabolic syndrome, type 2 diabetes, fertility, certain tumor types and increased mortality)<sup>[1-6]</sup>.

One of the major comorbidities of obesity is type 2 diabetes mellitus (T2DM). In fact, the term “diabesity”<sup>[7]</sup> has been introduced to refer to obesity accompanied by T2DM.

With the exception of nutritional and some pharmacological treatments, bariatric surgery is performed more and more frequently as the treatment of choice in patients with severe obesity.

The efficacy of these surgical procedures in weight control has been widely described in several studies. Additionally, one of the most relevant corollary effects reported following bariatric surgery is T2DM remission.

A variety of surgical procedures are available and, currently, it is difficult to identify the most effective option based on patient characteristics and comorbidities. Furthermore, little is known regarding the effect of the various surgical procedures on glycemic control and on T2DM remission<sup>[8-11]</sup>.

The aim of this study is to compare the clinical efficacy of laparoscopic sleeve gastrectomy (SG) and laparoscopic mini-gastric bypass (MGB) in terms of T2DM remission.

## MATERIALS AND METHODS

Data from patients referred during a 3-year period (from January 2009 to December 2011) to the University of Naples “Federico II” diagnosed with obesity and diabetes were retrieved from a prospective database.

Only patients who underwent uneventful laparoscopic SG or MGB with a follow-up of at least 1 year were included.

The patients were split into two groups according to the surgical intervention performed (SG and MGB).

Medical records were reviewed to collect the demographic and clinical characteristics of the patients. According to standard procedures<sup>[12]</sup>, the study population was stratified based on abdominal obesity [body mass index (BMI) > 30 kg/m<sup>2</sup>], triglycerides levels (equal to or

> 150 mg/dL), HDL-cholesterol (< 40 mg/dL for men and 50 mg/dL for women with total cholesterol values > 200 mg/dL), blood pressure (systolic blood pressure equal to or > 130 and/or diastolic > 85 mmHg) and fasting glucose levels (equal to or > 110 mg/dL).

Diagnosis of T2DM was made according to the American Diabetes Association guidelines. T2DM remission was defined as a fasting plasma glucose level below 126 mg/dL in the absence of hypoglycemic drugs.

The indications for treating these patients were the same as those published in the Italian Society for Bariatric Surgery guidelines<sup>[13]</sup>. Following the failure of a non-operative weight reduction program that included diet and other interventions (*e.g.*, behavioral modification, psychotherapy, dietary counseling, or physical training), bariatric surgery plays a critical role in patient outcome when the BMI is > 40 kg/m<sup>2</sup> or is > 35 kg/m<sup>2</sup> combined with serious co-existing conditions.

Only surgical procedures performed by an expert surgeon (more than 500 laparoscopic surgical procedures) were included in the analysis.

Prevention of surgical site infection and perioperative antiplatelet drug administration were managed according to validated criteria<sup>[14,15]</sup>.

For the sleeve gastrectomy procedure, 75%-80% of the greater curvature was excised, leaving a narrow stomach tube of 38F. Single-loop gastric bypass was performed, which consisted of constructing a 40-70 mL sleeve gastric pouch with a jejunal exclusion of 200-220 cm. All procedures were performed using a laparoscopic approach<sup>[16-18]</sup>.

Following surgery, clinical controls were performed once a month for the first 3 postoperative months and every 3 mo thereafter.

At each follow-up visit, weight loss and glycemic control status [blood glucose, hemoglobin A1c (HbA1c) and hypoglycemic treatment] were evaluated. Diabetes remission was defined as HbA1c values less than 6.5 without the use of oral hypoglycemic treatment or insulin<sup>[11,19]</sup>.

Statistical analysis was performed using the SPSS 17 system (SPSS Inc., Chicago, IL, United States). Continuous data were expressed as the mean  $\pm$  SD, and categorical variables were expressed as the percent changes. To compare continuous variables, an independent and/or paired sample *t* test was performed, and correlation was assessed using the Pearson's linear correlation coefficients (*r*). Changes in BMI, glycemia and HbA1c were expressed as the percent changes *vs* baseline values. The  $\chi^2$  test was used to analyze categorical data. When the minimum expected value was < 5, the Fisher's exact test was used. To adjust for major covariates and to generate predictions, a logistic regression (stepwise) model was applied, with diabetes remission at 12 mo as the dependent variable and gender, age, hypertension, hypercholesterolemia, current hypoglycemia treatment, BMI baseline value, glycemia and HbA1c as independent variables. All of the results are presented as 2-tailed values with statistical significance defined as *P* values < 0.05.

**Table 1** Baseline clinical and demographic characteristics of the study population *n* (%)

	Sleeve ( <i>n</i> = 15)	Mini-bypass ( <i>n</i> = 16)	<i>P</i> value
Age	37.26 ± 3.7	39.3 ± 2.3	0.076
Male gender	7 (46.7)	8 (50.0)	1.000
BMI	43.6 ± 2.99	45.8 ± 5.0	0.140
Diabetes treatment			
Metformin ± insulin	7 (46.7)	9 (56.3)	0.724
Metformin	8 (53.3)	7 (43.7)	
Glycemia	161.4 ± 31.4	177.8 ± 38.6	0.207
HbA1c	8.6 ± 1.0	8.47 ± 1.1	0.782
Hypertension	8 (53.3)	10 (62.5)	0.722
Diabetes	15 (100.0)	16 (100.0)	1.000
Hypercholesterolemia	4 (26.6)	4 (25.0)	1.000

BMI: Body mass index; HbA1c: Hemoglobin A1c.

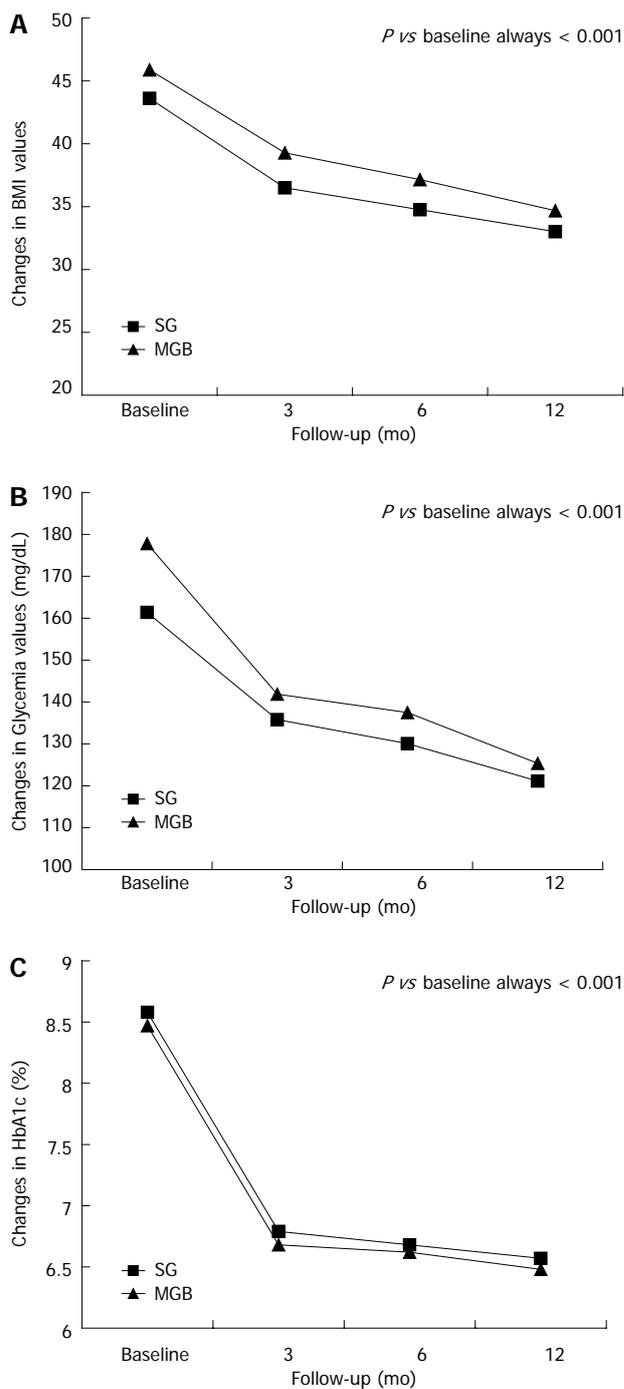
## RESULTS

A total of 53 subjects who underwent sleeve gastrectomy or mini-gastric bypass for obesity and diabetes were screened for the inclusion in this study. Of these, 4 subjects were excluded because of surgical complications, 7 subjects were omitted because young surgeons conducted the operations and 11 subjects were removed because of the lack of follow-up. Thus, a total of 31 obese patients (15 males and 16 females; mean age: 38.32 ± 3.21 years; BMI: 44.78 ± 4.25 kg/m<sup>2</sup>) were recruited for this study. All patients were diagnosed with type 2 diabetes [15 (48.4%) on metformin and 16 (51.6%) on metformin + insulin], 18 subjects (58.1%) reported hypertension and 8 presented with hypercholesterolemia. The mean glycemia value was 169.87 ± 35.76, and the mean HbA1c level was 8.5 ± 1.0. A total of 15 subjects underwent SG (48.4%), and 16 patients underwent MGB (51.6%). Major clinical and demographic characteristics of the study population stratified according to type of surgery are reported in Table 1.

After surgical intervention, a significant and consistent reduction in BMI, glycemia and HbA1c values were observed relative to the baseline values (Figure 1). Stratifying for type of surgery, SG and MGB were associated with similar percent changes in BMI (-24.33 ± 4.48 *vs* -24.19 ± 4.42, *P* = 0.931), glycemia (-24.30 ± 11.40 *vs* -28.42 ± 14.03, *P* = 0.379) and HbA1c (-22.57 ± 8.70 *vs* -22.67 ± 8.46, *P* = 0.975).

Overall, significant correlations were not detected in the percent change from baseline to 12-mo follow-up between BMI and glycemia, as well as between BMI and HbA1c (Figure 2). Additionally, the same results were confirmed after stratifying based on the type of surgery. Indeed, the percent change in BMI did not correlate with changes in glycemia (*r* = -0.119, *P* = 0.673 for SG and *r* = 0.462, *P* = 0.071 for MGB) or with changes in HbA1c (*r* = -0.349, *P* = 0.202 for SG and *r* = -0.018, *P* = 0.946 for MGB).

As shown in Figure 3, the prevalence of diabetes remission was gradually increased following surgery, regardless of the type.



**Figure 1** Changes in body mass index (A), glycemia (B) and hemoglobin A1c (C) values following surgery. HbA1c: Hemoglobin A1c; SG: Sleeve gastrectomy; MGB: Mini-gastric bypass; BMI: Body mass index.

Specifically, at 3 mo post-surgical intervention, diabetes remission was reported by 18 subjects (53.3% in SG *vs* 62.5% in MGB, *P* = 0.722). Similar results were confirmed at the 6-mo follow-up (53.3% for SG *vs* 68.8% for MGB, *P* = 0.473).

At the 12-mo follow-up, 66.7% of subjects who underwent SG achieved diabetes remission *vs* 87.5% of those who underwent MGB (*P* = 0.220).

Interestingly, the percent change in BMI was similar between patients achieving diabetes remission and

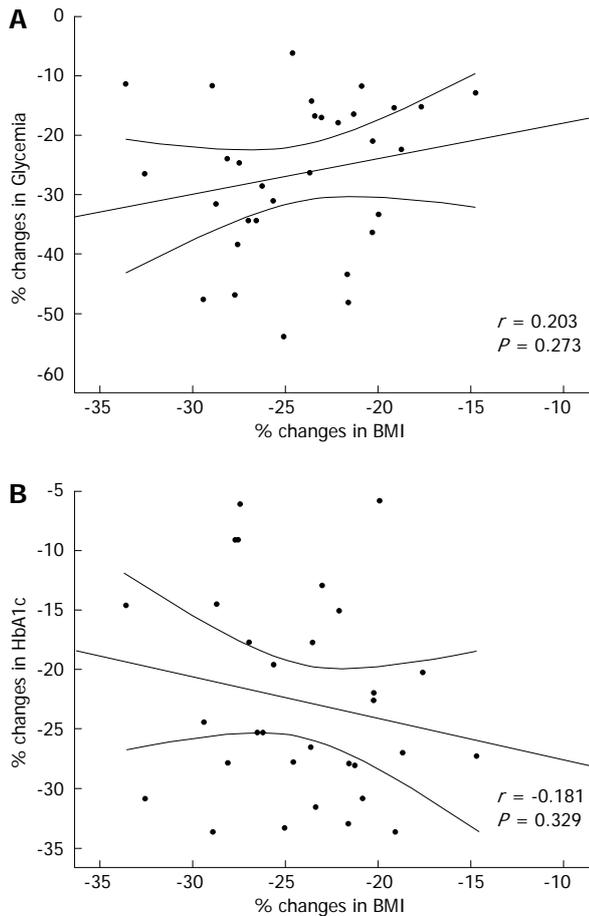


Figure 2 Scatter plot of Pearson's correlations between the percent change in glycemia and body mass index (A) and in hemoglobin A1c and body mass index (B) following surgical intervention. HbA1c: Hemoglobin A1c; BMI: Body mass index.

patients who did not ( $-24.28 \pm 4.33$  vs  $-24.15 \pm 4.53$ , respectively,  $P = 0.97$ ).

After adjusting for various clinical and demographic characteristics in a multivariate logistic regression analysis, a high HbA1c was considered a negative predictor of diabetes remission at 12 mo (OR = 0.366, 95%CI: 0.152-0.884). Using the same regression model, MGB showed a clear trend towards a higher diabetes remission rate relative to SG (OR = 3.780, 95%CI: 0.961-14.872).

## DISCUSSION

The prevalence of type 2 diabetes has markedly increased in the last decade, both in the United States<sup>[20-23]</sup> and globally<sup>[24-26]</sup>. These data are correlated with a comparably steep increase in the prevalence of obesity<sup>[27-29]</sup>. The primary risk factor for type 2 diabetes is obesity, and 90% of all patients with type 2 diabetes are either overweight or obese<sup>[30,31]</sup>. The National Health and Nutrition Examination Survey III (1988-1994) data demonstrated that the risk for chemical diabetes is approximately 50% with a BMI of greater than or equal to 30 kg/m<sup>2</sup> and over 90% with a BMI of 40 kg/m<sup>2</sup> or more<sup>[32]</sup>. The Nurses' Health Study that was conducted on 84941 women (1980-1996)

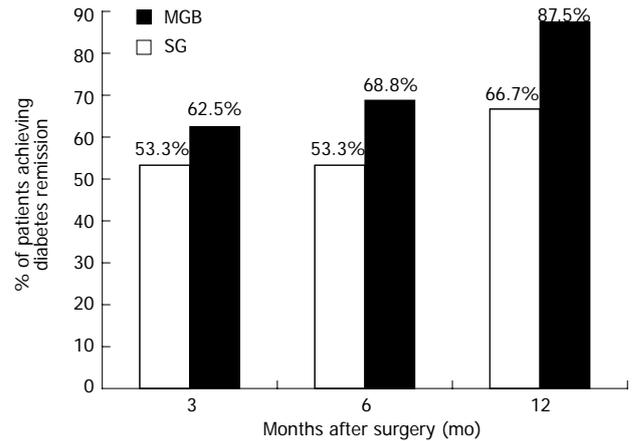


Figure 3 Prevalence of subjects achieving diabetes remission in the sleeve gastrectomy group and the mini-gastric bypass group. SG: Sleeve gastrectomy; MGB: Mini-gastric bypass.

showed that the relative risk of diabetes increased approximately 40-fold as the BMI increased from less than 23 kg/m<sup>2</sup> to more than 35 kg/m<sup>2</sup><sup>[33]</sup>.

Morbid obesity has been defined by the National Institutes of Health as a BMI of greater than or equal to 40 kg/m<sup>2</sup> or greater than or equal to 35 kg/m<sup>2</sup> in the presence of obesity comorbidities<sup>[13,19,20]</sup>.

The prevalence of people who are overweight or obese has increased dramatically in high-income countries during the past 20 years. The World Health Organization estimates that 54.3% of women and 51.7% of men in the United States will be obese (BMI 30 kg/m<sup>2</sup>) in 2015.

Obesity is notoriously difficult to manage. Diet, behavioral therapy, exercise, and pharmacologic intervention have traditionally been used but generally yield modest results. Additionally, weight regain is a common problem. In cases of failed medical therapy, bariatric surgery should be considered the treatment of choice for severe obesity<sup>[34]</sup>.

Surgical treatment of obesity is a rapidly growing area of surgical practice, reflecting the ability of bariatric surgery to achieve significant and durable weight loss, as well as the evolution of safer, less-invasive procedures<sup>[35-37]</sup>.

A remarkable effect of bariatric surgery is the profound and durable resolution of type 2 diabetes clinical manifestations. In a meta-analysis of 134 studies that reported comorbidity resolution (2738 citations), bariatric surgery followed by resolution of type 2 diabetes was observed in 48% of patients who underwent laparoscopic adjustable gastric banding, 84% of patients who underwent gastric bypass, and 98% of patients who underwent biliopancreatic diversion/duodenal switch<sup>[38]</sup>. These data were based on reports from 22094 patients from January 1, 1990 to June 5, 2003.

Roux-en-Y gastric bypass (RYGB) is considered the gold standard bariatric procedure for achieving diabetes remission.

To explain the mechanisms underlying the effective-

ness of gastric bypass procedures in normalizing glycemia, it has been suggested that removal of the gut may play a major role in diabetes remission, especially because important hormones are secreted from this region. In 2009, Cummings reviewed the existing hypotheses regarding the mechanisms underlying diabetes remission. Based on this review, the main hypotheses include the ghrelin hypothesis, the upper intestinal hypothesis and the lower intestinal hypothesis. The ghrelin hypothesis<sup>[39]</sup> maintains that ghrelin regulation may be disturbed following RYGB. Ghrelin is a hormone secreted by the stomach and proximal small bowel, particularly before meals. Its main physiological effects include increased appetite and fat mass increase<sup>[40]</sup>. In support of the ghrelin hypothesis, several studies have shown that ghrelin levels are very low following RYGB. Diminished ghrelin secretion can decrease appetite and food intake, and reduced secretion might also have a role in increasing glucose tolerance, as ghrelin can stimulate counter-regulatory hormones<sup>[41]</sup>. The lower intestinal hypothesis claims that intestinal shortcuts, created by bariatric surgery, expedite delivery of ingested nutrients and increase glucagon-like peptide-1 (GLP-1) release. GLP-1 is an incretin, defined as a peptide secreted from enteroendocrine L-cells. These cells are found throughout the small intestine and at a high density in the ileum. GLP-1 increases insulin secretion and has also been shown to increase proliferation and decrease apoptosis of beta-cells<sup>[42]</sup>. Both RYGB and BPD create gastrointestinal shortcuts, and studies have shown that postprandial GLP-1 secretion is increased post-surgery<sup>[43,44]</sup>. Therefore, it seems reasonable that, following surgery, GLP-1 secretion may be enhanced, leading to enhanced insulin secretion. This mechanism could also explain the increase in  $\beta$ -cell mass that is thought to accompany post-RYGB hyperinsulinemic hypoglycemia<sup>[45]</sup>. The upper intestinal hypothesis maintains that avoiding nutrient contacts with the duodenum is somehow key in the process through which diabetes is improved. The basis of this hypothesis is that unknown factors or processes from the duodenum influence glucose homeostasis<sup>[39]</sup>. Rubino and Marescaux<sup>[46]</sup> were the first to provide support for this hypothesis. They experimented on a variant of RYGB creating the intestinal bypass but leaving the stomach intact, which induced the same digestive discontinuation without re-anastomosis. This surgery, termed duodenal-jejunal bypass, was tested in several studies showing an improvement in T2DM without reduction in body weight<sup>[41]</sup>. These studies suggest that the exclusion of the proximal intestine may play a role in diabetes remission.

Interestingly, sleeve gastrectomy and mini-gastric bypass have emerged as new and effective weight loss procedures<sup>[8-11,16-18,47,48]</sup>.

There is increasing evidence indicating that SG causes early and significant improvements in glucose homeostasis in most morbidly obese subjects with T2DM<sup>[49-52]</sup>.

A systematic review of the existing literature showed

that SG results in T2DM resolution ranging from 80% to 96% in morbidly obese subjects<sup>[50]</sup>, a range similar to that in patients following RYGBP<sup>[38]</sup>.

Similarly, laparoscopic mini-gastric bypass is reported to be a safe alternative to LRYGB, showing comparable efficacy in weight reduction and resolution of metabolic complications, including diabetes<sup>[52-54]</sup>.

Both short-term<sup>[55-57]</sup> and long-term<sup>[58,59]</sup> follow-up confirmed the durable effect of this simplified procedure for obese or morbidly obese patients with T2DM.

Laparoscopic mini-gastric bypass in morbidly obese patients with T2DM has been shown to be effective in prospective randomized controlled trials<sup>[55]</sup>, as well as in extensive reports in the literature<sup>[54-59]</sup>.

Ghrelin regulation is disturbed following the sleeve gastrectomy procedure. SG was also reported to have a hindgut effect with increasing levels of glucagon-like peptide 1 and peptide YY due to the increased transit time after SG<sup>[60]</sup>.

Because construction of the sleeve gastric pouch is the first step of this technique, similar mechanisms are involved in mini-gastric bypass procedure.

The only region unaffected by SG was on the foregut. Specifically, the upper intestine was not in contact with ingested nutrients in the GB-treated group, while contact was made in the SG-treated group.

Recently, Lee *et al.*<sup>[9]</sup> published the first comparative study between sleeve gastrectomy and mini-gastric bypass to determine the efficacy of these treatments on diabetic control. Their results strongly support the hypothesis that duodenal exclusion may play a role in diabetes mellitus resolution following bariatric surgery in overweight patients.

Our findings extend the observations of Lee to severely obese patients. Unlike the study conducted by Lee *et al.*<sup>[9]</sup>, we only enrolled patients diagnosed with severe obesity and a clear indication to bariatric surgery. Despite this difference in the recruited patient population, our results also confirm that MGB is associated with better glycemic control and a higher rate of diabetes remission.

Although we observed a clear trend in our study, this did not achieve statistical significance. A multivariate analysis was performed to adjust for major clinical and demographic variables, but because of the relatively small sample size, our results need to be validated in larger studies. Thus, the present work could be considered a preliminary study, providing the rationale for a randomized prospective trial.

Further supporting this hypothesis, we reported that BMI changes were similar between patients achieving diabetes remission and patients not attaining remission. This finding suggests that diabetes remission may be independent from weight loss and that the type of surgery may play a more relevant role. These results combined with the evidence that weight loss is similar following the sleeve gastrectomy and mini-gastric bypass procedures may still support the theory suggested by Lee *et al.*<sup>[9]</sup>. Consequently, if we exclude the role of weight loss and

both the ghrelin and hindgut theories, the only remaining theory to explain the different results is the foregut (duodenal exclusion).

Overall, this mechanism suggests a potential superiority of the mini-gastric bypass over the sleeve gastrectomy in obtaining diabetes remission, but further data are needed to make this conclusion.

While the gold standard for diabetes remission remains the Roux-en-y gastric bypass because similar mechanisms of diabetes remission may be involved and the procedure is easier to perform, the mini-gastric bypass could become a valuable alternative.

Although our results appear to be encouraging and support mini-gastric bypass as an effective treatment strategy for diabetes remission, further studies are needed to allow for definitive conclusions regarding the ideal procedure for obtaining diabetes remission.

## COMMENTS

### Background

A remarkable effect of bariatric surgery is the profound and durable resolution of type 2 diabetes clinical manifestations. Interestingly, although both sleeve gastrectomy and mini-gastric bypass have emerged as new and effective weight loss procedures, the duodenal exclusion (involved in the mini-gastric bypass) may play a role in diabetes mellitus resolution.

### Research frontiers

The encouraging results obtained in this study with the mini-gastric bypass provide the rationale for a future randomized prospective trial to validate the effectiveness of this surgical technique.

### Innovations and breakthroughs

Their results confirm that mini-gastric bypass is associated with better glycemic control and higher diabetes remission rates relative to sleeve gastrectomy. The gold standard for diabetes remission remains Roux-en-y gastric bypass. However being similar mechanisms of diabetes remission involved and being easier to be performed, the mini-gastric bypass could become a valuable alternative.

### Applications

Mini-gastric bypass should be considered an effective weight loss procedure in diabetic patients.

### Peer review

The results are interesting and suggest that the effectiveness of mini-gastric bypass on diabetes remission, further studies are needed to provide definitive conclusions in selecting the ideal procedure for diabetes remission.

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## No evidence of HPV DNA in esophageal squamous cell carcinoma in a population of Southern Brazil

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### Abstract

**AIM:** To investigate the association between human papillomavirus (HPV) and esophageal squamous cell carcinoma (ESCC) in southern Brazil.

**METHODS:** We studied 189 esophageal samples from 125 patients from three different groups: (1) 102 biopsies from 51 patients with ESCC, with one sample from the tumor and another from normal esophageal mucosa distant from the tumor; (2) 50 esophageal biopsies from 37 patients with a previous diagnosis of head and neck squamous cell carcinoma (HNSCC); and

(3) 37 biopsies from esophageal mucosa with normal appearance from 37 dyspeptic patients, not exposed to smoking or alcohol consumption. Nested-polymerase chain reaction (PCR) with the MY09/11 and GP5/6 L1 primers was used to detect HPV L1 in samples fixed in formalin and stored in paraffin blocks. All PCR reactions were performed with a positive control (cervicovaginal samples), with a negative control (Human Genomic DNA) and with a blank reaction containing all reagents except DNA. We took extreme care to prevent DNA contamination in sample collection, processing, and testing.

**RESULTS:** The histological biopsies confirmed the diagnosis of ESCC in 52 samples (51 from ESCC group and 1 from the HNSCC group) and classified as well differentiated (12/52, 23.1%), moderately differentiated (27/52, 51.9%) or poorly differentiated (7/52, 13.5%). One hundred twenty-eight esophageal biopsies were considered normal (51 from the ESCC group, 42 from the HNSCC group and 35 from dyspeptic patients). Nine had esophagitis (7 from the HNSCC and 2 from dyspeptic patients). Of a total of 189 samples, only 6 samples had insufficient material for PCR analysis: 1 from mucosa distant from the tumor in a patient with ESCC, 3 from patients with HNSCC and 2 from patients without cancer. In 183 samples (96.8%) GAPDH, G3PDH and/or  $\beta$ -globin were amplified, thus indicating the adequacy of the DNA in those samples. HPV DNA was negative in all the 183 samples tested: 52 with ESCC, 9 with esophagitis and 122 with normal esophageal mucosa.

**CONCLUSION:** There was no evidence of HPV infection in different ESCC from southern Brazil.

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**Key words:** Esophageal cancer; Esophageal squamous cell carcinoma; Human papillomavirus; Head and neck cancer; Polymerase chain reactions; Nested-polymerase

chain reaction

**Core tip:** This paper gives additional evidence related to the controversy on the potential role of human papillomavirus (HPV) in the pathogenesis of esophageal squamous cell carcinoma (ESCC). Taking great care to avoid contamination by environmental HPV and using a very sensitive HPV DNA detection technique, we found no evidence of HPV neither in ESCC tumor tissue, nor in esophageal non-tumoral tissue from ESCC patients, or from head and neck squamous cell carcinoma patients or from dyspeptic controls without cancer. These data convincingly argue that when environmental contamination is carefully controlled, there is no evidence that HPV is involved in ESCC carcinogenesis in southern Brazil.

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## INTRODUCTION

Worldwide, esophageal cancer is the eighth most common cancer and the sixth most common cause of death from cancer, with an estimated incidence of 482000 new cases and 407000 deaths in 2008<sup>[1]</sup>. Esophageal squamous cell carcinoma (ESCC) is still the most common type worldwide and its known risk factors are smoking and excessive alcohol consumption, poor nutritional and socio-economic status, exposure to polycyclic aromatic hydrocarbons (PAH), low consumption of fruits and vegetables, ingestion of hot beverages, genetic factors, history of caustic injury in the esophagus, and history of head and neck squamous cell carcinoma (HNSCC)<sup>[2-4]</sup>. In Brazil, the highest rates of esophageal cancer occur in the country's southernmost state, Rio Grande do Sul, where the rate of incidence is considered moderate with 18.01 cases per 100000 men and 6.60 cases per 100000 women<sup>[5]</sup>. In the south of Brazil the most important risk factors are the combination of smoking and excessive alcohol consumption<sup>[5]</sup>, however, the consumption of a hot beverage made with the infusion of *Ilex paraguayensis* (also known as *yerba mate*) is also a risk factor<sup>[6]</sup>. This beverage is often consumed at high temperatures and contains high levels of PAH<sup>[7]</sup>.

The role of human papillomavirus (HPV) in the development of ESCC remains controversial<sup>[8-12]</sup>. Two studies conducted in southern Brazil, each using a different technique, showed different results regarding the association between ESCC and HPV<sup>[13,14]</sup>. Therefore, to clarify the association between HPV infection and ESCC in southern Brazil, we looked for the presence of HPV DNA in esophageal biopsies from: (1) primary tumor

and mucosa without neoplasia from patients with ESCC; (2) Lugol stained and unstained areas in patients with HNSCC; and (3) normal appearing mucosa from patients not exposed to tobacco or alcohol.

## MATERIALS AND METHODS

### Patients and tissues

We evaluated 189 consecutive esophageal samples, collected in Santa Maria, a city in the central region of Rio Grande do Sul, the most southern state in Brazil, from 2008 to 2011. We included 51 samples from esophageal tumors and 51 samples from non-tumoral areas of the esophagus of patients with ESCC. We analyzed 50 esophageal biopsies (32 from Lugol stained areas, 17 from unstained areas and 1 from tumor) from patients diagnosed with HNSCC. Neither the patients with ESCC nor those with HNSCC had received chemotherapy or radiotherapy before the sample collection. We also collected biopsies from the normal middle esophagus of thirty-seven non-smoking and non-alcohol drinking dyspeptic patients who underwent upper GI endoscopy.

We collected demographic data (sex, age, place of birth, occupation) and information regarding smoking habits, alcohol consumption and previous history of cancer. The samples were fixed in neutral buffered formalin, embedded in paraffin, cut and stained with Hematoxylin-Eosin. For DNA extraction, one slice at least 5  $\mu$ m thick, as recommended for polymerase chain reaction (PCR) amplification, was cut from the paraffin-embedded tissues<sup>[15]</sup>. To minimize the risk of contamination, the materials used to process the sample stored in the paraffin block were completely disposable and used once per sample. In addition, the microtome sample holder was washed with absolute alcohol and the blade was replaced before each block was cut. Different rooms were used for DNA extraction, preparing the DNA solution, adding the DNA samples to the PCR solution and electrophoresis analysis. The rooms could only be accessed through antechambers with a single flow of material. All PCR reactions were performed with a positive control (cervicovaginal samples), with a negative control (Human Genomic DNA, Cat. No. G304A, Promega, Madison, WI) and with a blank reaction containing all reagents except DNA. The same method was also employed to test for the presence of HPV DNA in thirty-five samples of primary tumor from patients with HNSCC.

### DNA Extraction and PCR

Once the samples were de-waxed, the DNA was extracted using the Qiagen QIAamp DNA Mini Kit (Valencia, CA) according to manufacturer's instructions. DNA quantification and purity were determined by optical density in a spectrophotometer (Thermo Scientific NanoDrop 2000).

The DNA (25-80 ng) from each sample was amplified by PCR. The integrity of the DNA samples was observed by amplifying the human conserved genes GAP-

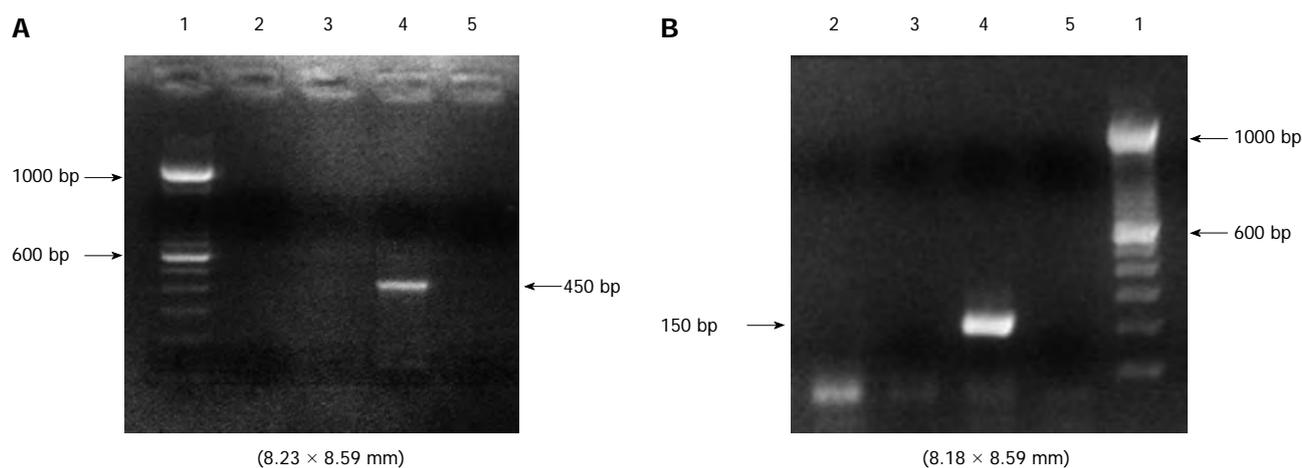


Figure 1 Analysis of DNA from esophageal tumor tissue for human papillomavirus using nested- polymerase chain reaction for human papillomavirus L1 gene. The MY09/11 primer pair was used in the first step (A) and the GP5/GP6 primer pair was used in the second step (B). 1: DNA size marker (100 bp DNA ladder); 2: Patient tumor DNA: [human papillomavirus (HPV)]-negative; 3: HPV DNA-negative control (Human Genomic DNA); 4: HPV DNA-positive control (cervicovaginal sample); 5: Negative control containing all polymerase chain reaction reagents except DNA.

DH, G3PDH and  $\beta$ -globin. The sequences of the *HPV L1* gene were amplified by nested-PCR using two general primer sets: MY09/MY11 (MY09: 5'-GTCCMARRG-GAWACTGATC-3', MY11: 5'-GCMCAGGGWCATA-AYAATGG-3') in the first amplification step to produce a 450 bp fragment; and GP5/GP6 (GP5: 5'-TTTGT-TACTGTGGTAGATAC-3', GP6: 5'-ACTAAATGT-CAAATAAAAAG-3') in the second step to produce 150 bp fragments of the PCR product. In the first step, PCR was performed with a reaction mixture containing 50  $\mu$ L, including 5.0  $\mu$ L of the genome from the extracted DNA sample, 5.0  $\mu$ L 10  $\times$  PCR buffer, 5.0  $\mu$ L of dNTP (2.5 mmol/L), 1.2  $\mu$ L of MgCl<sub>2</sub> (50 mmol/L), 0.5  $\mu$ L of Taq DNA polymerase and 0.2  $\mu$ L (500 pmol/ $\mu$ L) of the MY09 and MY11 primers. In the second step, which also had a final volume of 50  $\mu$ L, 1.0  $\mu$ L (500 pmol/ $\mu$ L) of the GP5 and GP6 primers was used. The PCR mixture was subjected to 40 amplification cycles, each consisting of an initial denaturation step at 94  $^{\circ}$ C for 30 s, annealment at 56  $^{\circ}$ C for 1 min and extension at 72  $^{\circ}$ C for 1 min. The PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide by electrophoresis (Figure 1).

### Ethics

The study was approved by the Research Ethics Committee of the Federal University of Santa Maria and of the Federal University of Rio Grande do Sul, Rio Grande do Sul, Brazil. Informed consent was obtained from each participant before they underwent upper GI endoscopy.

### Statistical analysis

The variables were expressed as mean and SD or numbers and percents. Associations would have been considered statistically significant when a two-sided *P* value was  $\leq 0.05$ . All statistical analyzes were performed with the aid of SPSS 11 (Statistical Package for Social Sciences).

## RESULTS

### Patient characteristics

We included 125 individuals divided in three groups (Table 1): (1) 51 patients with ESCC, 43 male (84.3%) with a mean age of  $60.1 \pm 10.3$  years; (2) 37 patients with previous diagnosis of HNSCC, 34 males (91.9%) with a mean age of  $57.8 \pm 8.4$  years; and (3) 37 dyspeptic patients, 12 males (32.4%) with a mean age of  $56.8 \pm 17.7$  years.

### Histology

We studied 189 esophageal biopsy samples. All fifty-two biopsies from tumoral areas (51 from the ESCC group and 1 from the HNSCC group) were diagnosed as squamous cell carcinoma and classified as well differentiated (12/52, 23.1%), moderately differentiated (27/52, 51.9%) or poorly differentiated (7/52, 13.5%). None of the 51 samples of esophageal mucosa distant from the tumor in the ESCC group showed malignancy. In the patients with HNSCC, we performed 55 esophageal biopsies. Their histological analysis showed the following findings: 42 were normal (76.4%), 7 had esophagitis (12.7%), 1 contained ESCC (1.8%) and five had insufficient tissue for histological analysis (9.1%). In the biopsies of normal esophagus in the dyspeptic patients, 35 were considered normal and 2 had mild esophagitis.

### PCR analysis

The average DNA concentration in the samples was 214.68 ng/ $\mu$ L (range: 8-1313), with a mean ratio between the absorbance readings at 260 nm and 280 nm wavelengths of 2.12 (range 1.15-5.95). GAPDH, G3PDH and/or  $\beta$ -globin were amplified in 183 (96.8%) esophageal biopsies, showing that the DNA was adequate for analysis. These conserved human genes were not amplified in only six samples, all with normal esophageal mucosa (1 from mucosa distant from the tumor in a patient

**Table 1 Clinical and demographic characteristics and risk factors of the patients *n* (%)**

	ESCC ( <i>n</i> = 51)	HNSCC ( <i>n</i> = 37)	Not exposed ( <i>n</i> = 37)
Age (yr)			
Range	42-79	41-78	19-87
mean ± SD	60.1 ± 10.3	57.8 ± 8.4	56.8 ± 17.7
Sex			
Male	43 (84.3)	34 (91.9)	12 (32.4)
Female	8 (15.7)	3 (8.1)	25 (67.6)
Smoking			
Current	42 (82.4)	35 (94.6)	-
Ex-smokers (> 10 yr)	5 (9.8)	2 (5.4)	10 (27.0)
Never smoked	4 (7.8)	0 (0)	27 (73.0)
Alcohol use			
Current	27 (53.0)	31 (83.8)	-
Ex-alcohol users (> 10 yr)	8 (15.7)	2 (5.4)	2 (5.4)
Never used alcohol	16 (31.4)	4 (10.8)	35 (94.6)
Active smokers and alcohol users	18 (35.3)	18 (48.6)	-
Never smoked or drunk alcohol	3 (5.9)	0 (0)	26 (70.3)

ESCC: Esophageal squamous cell carcinoma; HNSCC: Head and neck squamous cell carcinoma.

with ESCC, 3 from patients with HNSCC and 2 from patients without cancer). The PCR results were negative for HPV DNA in all the esophageal biopsies (52 with ESCC, 9 with esophagitis and 122 with normal esophageal mucosa), as well as in all biopsies from the primary tumor of head and neck cancer.

## DISCUSSION

The current study used a nested primer-based PCR test to identify HPV DNA in esophageal samples from individuals from a moderate risk area for ESCC in southern Brazil. Our results showed no evidence of HPV DNA in any of the samples of the ESCC and from non-tumoral areas of the esophagus, in the esophageal mucosa of patients with HNSCC, or in the esophageal mucosa from patients without risk factors for ESCC.

Some studies using a bovine model have found that the bovine papillomavirus is essential in the early stages of carcinogenesis of the upper digestive tract, but is not required for progression to the status of malignancy<sup>[8,16,17]</sup>. Considering the possibility of this “hit-and-run” mechanism to induce oncogenesis in the esophagus in humans, we included samples with and without cancer from the esophageal mucosa of patients with ESCC and esophageal mucosal samples from patients with HNSCC. In order to enhance the detection of ESCC precursor lesions, all patients with HNSCC underwent upper endoscopy with mucosal iodine staining and biopsy of stained and unstained areas<sup>[18-21]</sup>. However, all samples were negative for HPV DNA, both in the primary tumors and in the esophageal mucosa without neoplasia of patients with ESCC or HSNCC and in the group without cancer who were not exposed to tobacco or alcohol. The consistent presence of the GAPDH, G3PDH or  $\beta$ -globin genes in

the samples indicated that the specimens were suitable for DNA analysis.

We used the nested primer-based PCR system with the MY11-MY09 consensus primers in combination with another general primer pair, GP5-GP6, positioned within the former. According to Evander *et al*<sup>[22]</sup>, this two-step PCR amplification is able to detect 1 to 10 copies of the HPV 16 genome, while the use of isolated pairs of primers MY11-MY09 or GP5-GP6 detect 100 and 10 copies, respectively. Thus, we used a technique with good yield for samples with low viral load. In addition, the MY11-MY09 primer is highly capable of detecting multiple HPV types within a given sample<sup>[23]</sup>. Furthermore, the inner primer pair GP5-GP6 spans a shorter region (about 150 bp), which is useful when the DNA is isolated from paraffin-embedded tissue fixed with formalin<sup>[24-27]</sup>. More sensitive techniques can be used to detect HPV DNA, but as Ha *et al*<sup>[28]</sup> suggest, without at least one copy of the viral genome per cell, a clonal relationship cannot be established; therefore, a sufficient number of copies of HPV affecting most of the cells in the lesion is required. Hence, we believe that our technique was sufficiently sensitive to accurately detect the presence of HPV DNA in tissue samples from the aero-digestive tract.

In southern Brazil, Weston and Prolla<sup>[13]</sup> analyzed 40 ESCC samples and 10 benign esophageal biopsies from patients without cancer. Using the Hybrid Capture II test, they detected HPV DNA in only one case of ESCC and in one benign specimen. Subsequently, Souto Damin *et al*<sup>[14]</sup> reported detecting HPV in 15.75% of ESCC patients (26/165) and in none of the specimens of benign esophagus (0/26) using auto-nested PCR with the GP5+/GP6+ consensus primer pair. Some features of our study that differed from these two previous HPV studies conducted in southern Brazil are: (1) the extreme care we took to prevent DNA contamination throughout the specimen processing and testing; (2) the use of a more highly sensitive HPV detection method; and (3) the assessment of possible precursor lesions in the non-tumoral mucosa of esophagi with cancer and in the esophageal mucosa of patients with head and neck cancer.

A tissue study similar to ours was recently reported from a high-risk area of China<sup>[29]</sup>. The authors analyzed tumor samples from 272 patients with ESCC who underwent esophagectomy at the Yaocun Commune Hospital in Linxian, in north-central China. The patients came from various regions of China, with different incidence rates of ESCC and different mortality rates for cervical cancer. HPV DNA was tested on fresh frozen tumor tissue and tumor samples fixed with formalin and stored in paraffin using PCR with the PGMY L1 and SPF10 L1 consensus primers, respectively. Adopting careful measures to avoid contamination of the samples, similar to those used in the present study, these authors also found no cases with convincing evidence of carcinogenic HPV activity.

There are some limitations to the present study, such as the lack of information regarding sexual habits, socio-

economic characteristics and the consumption of the beverage *yerba mate*, but there is no reason to think that having such information would have changed the PCR results of this study.

Our results confirm previous observations in other regions that report the absence of an association between esophageal mucosa infection by HPV and ESCC, and suggest that, in southern Brazil, this virus is not an important risk factor for squamous cell carcinoma of the esophagus.

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## COMMENTS

### Background

The high geographical variation in esophageal squamous cell carcinoma (ESCC) incidence observed worldwide reflects the exposure to specific environmental factors that are not completely understood. High-risk human papillomavirus (HPV) infections are present in almost all cervical cancers, beside other neoplasms in squamous epithelial-lined tissues, probably being involved in their genesis. The etiologic role of HPV in ESCC remains highly controversial.

### Research frontiers

A large number of controversial manuscripts have been published investigating the possible role of HPV in the etiology of esophageal cancer. In this study the authors used a very sensitive HPV DNA detection technique, and evaluated the HPV presence in ESCC tumor tissue and esophageal non-tumoral tissue.

### Innovations and breakthroughs

Previous studies have suggested that the HPV would have an essential role in the early stages of the ESCC. Besides ESCC tumor tissue, they also examined esophageal non-tumoral tissue from the same ESCC patients. The authors also analysed esophageal biopsies from patients with head and neck squamous cell carcinoma and from dyspeptic controls without cancer. They used a highly sensitive technique for detection of HPV with nested-polymerase chain reaction (PCR), taking the utmost care to avoid false positive results caused by samples contamination.

### Applications

This study adds more evidence that HPV is not involved in esophageal carcinogenesis, and it also suggests the high prevalence of HPV in some ESCC studies can be due to contamination. Therefore, the results of this study reinforce the need of utmost care to avoid contamination in further protocols looking for HPV in tissue samples.

### Terminology

Nested PCR uses two sequential sets of primers. The first pair of PCR primers amplifies a fragment similar to a standard PCR. The second primer set binds to sequences in the target DNA that are within the portion amplified by the first set. Thus, the second set of primers will bind and amplify target DNA within the products of the first reaction. The advantage of nested PCR is that if the wrong PCR fragment was amplified, the probability is quite low that the region would be amplified a second time by the second set of primers.

### Peer review

Overall, it is a well written and easy to follow paper that reports a hot topic about the involvement of HPV infection in the development of ESCC. The technique and the sampling procedure used are correct. The topic of the con-

tribution of HPV to ESCC is an important one since there is a vaccine for HPV that if related to squamous cell carcinoma could have a significant impact on the public health implications of this disease. This is a well prepared manuscript that reads well.

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## Effects of 5-HT2B, 5-HT3 and 5-HT4 receptor antagonists on gastrointestinal motor activity in dogs

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antagonists on normal gastrointestinal motor activity were analyzed.

**RESULTS:** 5-HT2B, 5-HT3 and 5-HT4 receptor antagonists had no contractile effect on the fasting canine terminal ileum. The 5-HT3 and 5-HT4 receptor antagonists inhibited phase III of the interdigestive motor complex of the antrum and significantly inhibited colonic motor activity. In the proximal colon, the inhibitory effect was dose dependent. Dose dependency, however, was not observed in the distal colon. The 5-HT2B receptor antagonist had no contractile effect on normal colonic motor activity.

**CONCLUSION:** The 5-HT3 and 5-HT4 receptor antagonists inhibited normal colonic motor activity. The 5-HT2B receptor antagonist had no contractile effect on normal colonic motor activity.

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**Key words:** 5-hydroxytryptamine receptor antagonist; Colonic motility; Irritable bowel syndrome

### Abstract

**AIM:** To study the effects of 5-hydroxytryptamine (5-HT) receptor antagonists on normal colonic motor activity in conscious dogs.

**METHODS:** Colonic motor activity was recorded using a strain gauge force transducer in 5 dogs before and after 5-HT2B, 5-HT3 and 5-HT4 receptor antagonist administration. The force transducers were implanted on the serosal surfaces of the gastric antrum, terminal ileum, ileocecal sphincter and colon. Test materials or vehicle alone was administered as an intravenous bolus injection during a quiescent period of the whole colon in the interdigestive state. The effects of these receptor

**Core tip:** Previous studies have investigated the effects of 5-hydroxytryptamine (5-HT) receptor antagonists on abnormal colonic motor activity, following either the administration of 5-HT or stress induction. This study is the first to investigate the effects of 5-HT receptor antagonists on normal colonic motor activity in dogs. 5-HT3 and 5-HT4 receptor antagonists inhibited normal colonic motor activity. A 5-HT2B receptor antagonist had no contractile effect on normal colonic motor activity. 5-HT3 and 5-HT4 receptor antagonists may also be used as premedications for colonoscopy. A 5-HT2B receptor antagonist may be used for the treatment of diarrhea-predominant irritable bowel syndrome without the side effect of constipation.

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## INTRODUCTION

Over 95% of the 5-hydroxytryptamine (5-HT) in the body is found in the gastrointestinal tract; 90% of the gastrointestinal 5-HT is contained within enterochromaffin cells<sup>[1,2]</sup>, and the remaining 10% is contained within the enteric neurons<sup>[3,4]</sup>. Seven major types and multiple subtypes of 5-HT receptors have been identified. 5-HT receptors that are known to affect gut motor functions are those belonging to the 5-HT<sub>1</sub>, 2, 3, 4 and 7 subtypes<sup>[5]</sup>. 5-HT has a variety of actions; it can cause smooth-muscle contraction (*via* cholinergic nerve stimulation) or relaxation (*via* stimulation of inhibitory nitric oxide-releasing neurons)<sup>[6]</sup>. Mucosal release of 5-HT stimulates both intrinsic sensory neurons (most likely *via* 5-HT<sub>4</sub> receptors) and extrinsic sensory neurons (*via* 5-HT<sub>3</sub> receptors); these actions modulate sensation<sup>[7]</sup>. Previous studies have shown that 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor agonists stimulate gastrointestinal motility<sup>[8-12]</sup>. However, the effects of these antagonists are unknown.

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder associated with altered motility, secretion and visceral sensation<sup>[5]</sup>. Stress is known to strongly contribute to the pathogenesis of IBS<sup>[13]</sup>. Data have shown that systemic administration of 5-HT<sub>3</sub> receptor antagonists abolishes the abnormal contractions and visceral sensations induced by stress<sup>[14-16]</sup>, leading to the use of 5-HT<sub>3</sub> receptor antagonists in the treatment of diarrhea-predominant IBS. However, the effects of 5-HT<sub>3</sub> receptor antagonists on normal colonic motor activity are controversial. Previous studies have shown that 5-HT<sub>3</sub> antagonists have no effect on colonic motor activity during the interdigestive state in dogs and humans<sup>[17-19]</sup>. By contrast, the migrating motor complex in the murine small and large bowel is abolished by 5-HT<sub>3</sub> antagonist activity<sup>[20]</sup>. Ondansetron, a 5-HT<sub>3</sub> receptor antagonist, has been shown to delay colonic transit time in healthy subjects<sup>[21-23]</sup> and tends to slow left-sided colonic transit in patients with diarrhea-predominant IBS<sup>[24]</sup>. The effect of 5-HT<sub>3</sub> receptor antagonists on normal colonic motor activity is still unknown. However, these studies suggest that 5-HT<sub>3</sub> receptor antagonists may have an inhibitory effect on colonic motor activity.

5-HT<sub>2B</sub> receptors are expressed by the smooth muscle of the adult human gut<sup>[25]</sup>, but their functions are unclear. It is known that 5-HT<sub>2B</sub> receptor antagonists inhibit visceral hypersensitivity<sup>[26]</sup>. These data suggest that 5-HT<sub>2B</sub> receptor antagonists also have a potential

therapeutic role in the treatment of IBS. The effects of 5-HT<sub>2B</sub> receptor antagonists on normal colonic motor activity *in vivo* remain unknown.

5-HT<sub>4</sub> receptors are distributed along the gut, where they may play a role in mediating smooth muscle tone, peristaltic reflex and mucosal secretion. In healthy humans, 5-HT<sub>4</sub> receptor agonists have been demonstrated to stimulate both whole gut transit and colonic transit. Clinically, 5-HT<sub>4</sub> receptor agonists are used for patients suffering from gastro-esophageal reflux disease, dyspepsia or constipation-predominant IBS<sup>[5,27,28]</sup>. As with the 5-HT<sub>3</sub> receptors, the effects of 5-HT<sub>4</sub> receptors are complex. 5-HT<sub>4</sub> receptors mediate both the relaxation and contraction of circular smooth muscle<sup>[28-31]</sup>. The effect of 5-HT<sub>4</sub> receptor antagonists on normal colonic motor activity remains unknown.

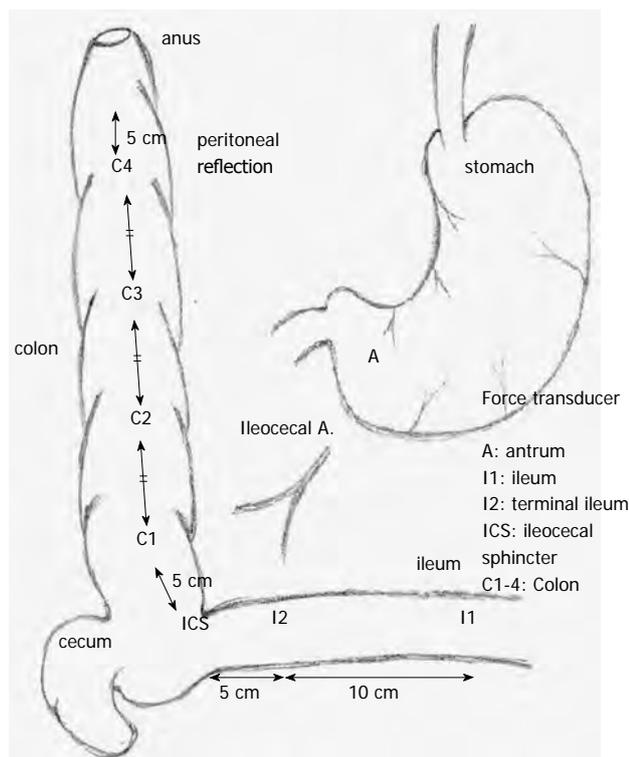
This study aimed to determine the effects of 5-HT<sub>2B</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists on normal colonic motor activity in conscious dogs.

## MATERIALS AND METHODS

### Preparation of animals

Experiments were completed in 5 healthy conscious dogs of both sexes, each weighing 8-11 kg. The procedures were approved by the Review Committee on Animal Use of Gunma University, Maebashi, Japan. Overnight-fasted dogs were anesthetized by a single intravenous injection of thiopental sodium (Ravonal; Tanabe Pharmaceutical, Osaka, Japan) at a dose of 20 mg/kg. General anesthesia was maintained by endotracheal inhalation of halothane (Fluothane; Takeda Chemical Industries, Osaka, Japan) and oxygen. A Silastic tube (Silastic 602-205; Dow Corning, Midland, MI) was inserted into the superior vena cava through a branch of the right internal jugular vein (jugular tube). The abdominal cavity was opened, and eight force transducers<sup>[32]</sup> were implanted on the serosal surfaces of the gastric antrum, terminal ileum (5 and 15 cm proximal to ileocecal sphincter) (I1/I2), ileocecal sphincter (ICS) and colon (C1-C4); C1 was placed 5 cm distal to the ICS, and C4 was placed 5 cm proximal to the peritoneal reflection. C2 and C3 were placed at equal distances between C1 and C4 (Figure 1). The ICS was identified by inspection and palpation. Wires from each force transducer were tunneled subcutaneously to the dorsum and connected to an eight-channel telemeter (GTS-800; Star Medical, Tokyo, Japan); gastrointestinal and colonic contractile activities were thereby continuously recorded on a computer (Adif1412.dill; Star Medical).

After the operation, the dogs were housed in individual experimental cages. They were fasted for 2 d after this procedure and maintained by intravenous infusion of Lactec G (Otsuka Pharmaceutical, Tokyo, Japan) at a daily volume of 500 mL. Cefmetazole (1 g) was administered intravenously, once preoperatively and once on postoperative day 1. The dogs were allowed to recover for  $\geq 10$  d. They were fed normal dog food (20 g/kg; Funabashi Farm, Funabashi, Japan) once daily and pro-



**Figure 1** Scheme of the dog model and the locations of the force transducers implanted in the canine gastrointestinal tract. Force transducers were implanted in the colon on the serosal surfaces of the gastric antrum (A), terminal ileum [5 and 15 cm proximal to ileocecal sphincter (ICS); I1-I2], ICS and colon (C1-C4); C1 was placed 5 cm distal to the ICS and C4 5 cm proximal to the peritoneal reflection. C2 and C3 were implanted equidistantly between C1 and C4.

vided water ad libitum.

After all experiments were completed, the dogs were sacrificed by an overdose of potassium chloride. Specimens of ICS were then fixed in 10% formalin and stained with hematoxylin and eosin. Proper placement of the transducers was confirmed.

### Drugs

Ondansetron, a 5-HT<sub>3</sub> receptor antagonist, was purchased from Sigma Japan (Tokyo). GR113808, a selective 5-HT<sub>4</sub> receptor antagonist<sup>[33]</sup>, was purchased from Wako Pure Chemical Co. (Osaka, Japan). RQ-00310941, a novel, potent, and selective 5-HT<sub>2B</sub> receptor antagonist<sup>[34]</sup>, was synthesized by RaQualia Pharma Inc. (Aichi Japan). Ondansetron was dissolved in distilled water, GR113808 was dissolved in DMSO, and RQ-00310941 was dissolved in acid DMSO.

### Experimental protocol and recording of contractile activity

The dogs were fasted overnight before each experiment. After the interdigestive motor complex<sup>[35]</sup> had been recorded at the antrum,  $\geq 2$ -h of contractile activity was recorded. Test material or vehicle (5 mL of 154 mmol/L NaCl solution) alone was then administered as an intravenous bolus injection during a quiescent state of the

whole colon in the interdigestive state. The experiments were performed with the following treatment doses: ondansetron (0.01, 0.03, 0.10 and 0.30 mg/kg), GR 113808 (0.1 and 0.3 mg/kg), and RQ-00310941 (1, 3 and 10 mg/kg). Contractile activities were recorded for  $\geq 2$ -h after administration. All experiments were carried out in a random order, and the test material was given once per day.

### Data analysis

The recorded mechanical activities were analyzed using software for analysis of gastrointestinal motility (8STAR, Star Medical Co., Ltd). The analysis of colonic contractile activity (from ICS to C4) was performed for 1 h both before and after administration. The mean motility index (MI) and average MI of colonic contractile activity were recorded at each site. The MI was defined as the integrated area between baseline (zero level) and the contractile wave expressed in motor units. This parameter was expressed as the inhibition ratio: the ratio of contractile activity before and after drug administration.

### Statistical analysis

Student's *t* test was used for statistical analysis. The results are expressed as the mean  $\pm$  SD. *P* < 0.05 was considered statistically significant.

## RESULTS

### Effects of 5-HT<sub>2B</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists on gastric and terminal ileum motility

5-HT<sub>2B</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists had no contractile effect on the fasted canine terminal ileum (I1, I2) during the observation period (Figures 2-5). In contrast, the 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists both significantly inhibited phase III of the interdigestive motor complex of the antrum (Figures 4 and 5).

### Effects of 5-HT<sub>2B</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists on normal colonic motor activity

The 5-HT<sub>2B</sub> receptor antagonist had no contractile effect on normal colonic motor activity (Figure 3). However, both the 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists inhibited colonic motor activity (Figures 4 and 5). At 0.3 mg/kg, the 5-HT<sub>3</sub> receptor antagonist significantly inhibited whole colonic motor activity (ICS - C4) (72%  $\pm$  9% *vs* -11%  $\pm$  6%, 70%  $\pm$  10% *vs* 6%  $\pm$  10%, 65%  $\pm$  15% *vs* 1%  $\pm$  11%, 61%  $\pm$  7% *vs* 5%  $\pm$  9% and 68%  $\pm$  7% *vs* -10%  $\pm$  3%, respectively; Figure 4). In the proximal colon, the inhibitory effects of the 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists were dose dependent. However, no dose-dependent response was observed in the distal colon. Upon administration of the 5-HT<sub>4</sub> receptor antagonist, colonic migrating motor complexes (CMMCs) corresponding to phase III of the migrating motor complex at the ileum were sometimes observed (Figure 5, see arrow). The motor complex independent of phase III of the migrating motor complex tended to be inhibited (Figure 5). These inhibited effects were observed for at least 1 h.

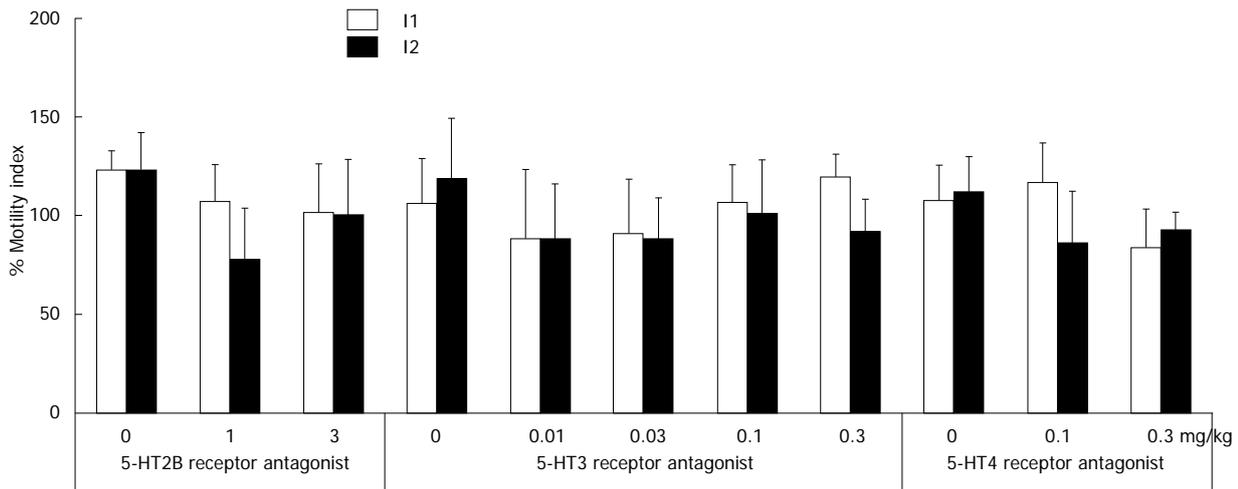


Figure 2 Inhibition ratio based on comparing the drug effects to before 5-hydroxytryptamine receptor antagonist administration in the terminal ileum (I1-I2). A systematic change was not observed. Values are mean  $\pm$  SE. 5-HT: 5-hydroxytryptamine; I1:Ileum; I2: Terminal ileum.

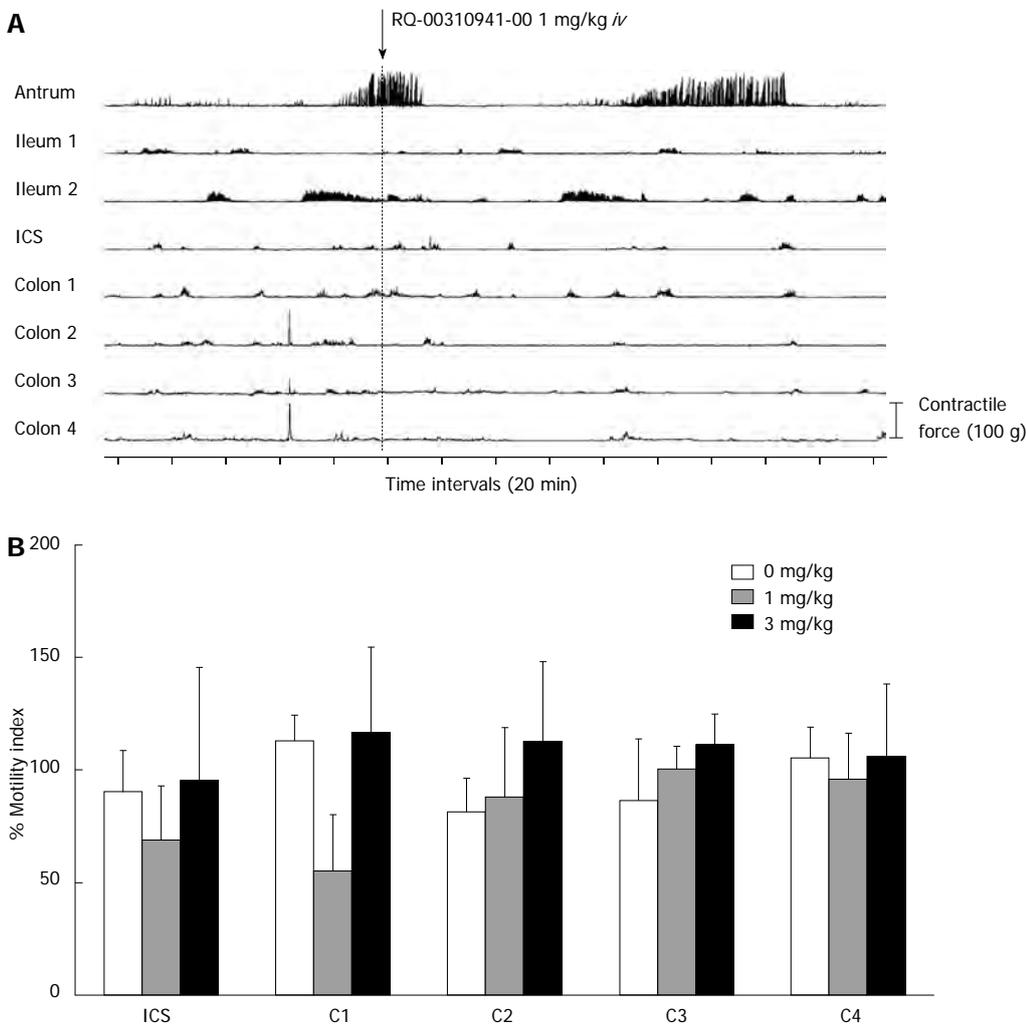


Figure 3 5-hydroxytryptamine 2B receptor antagonists on normal colonic motor activity. A: Typical effect of the 5-hydroxytryptamine (5-HT) 2B receptor antagonist. The 5-HT2B receptor antagonist had no contractile effect on the whole intestine; B: The inhibition ratio compared with before 5-HT2B receptor antagonist administration. A systematic change was not observed. Values are mean  $\pm$  SE. ICS: ileocecal sphincter; C1-4: Colon 1-4.

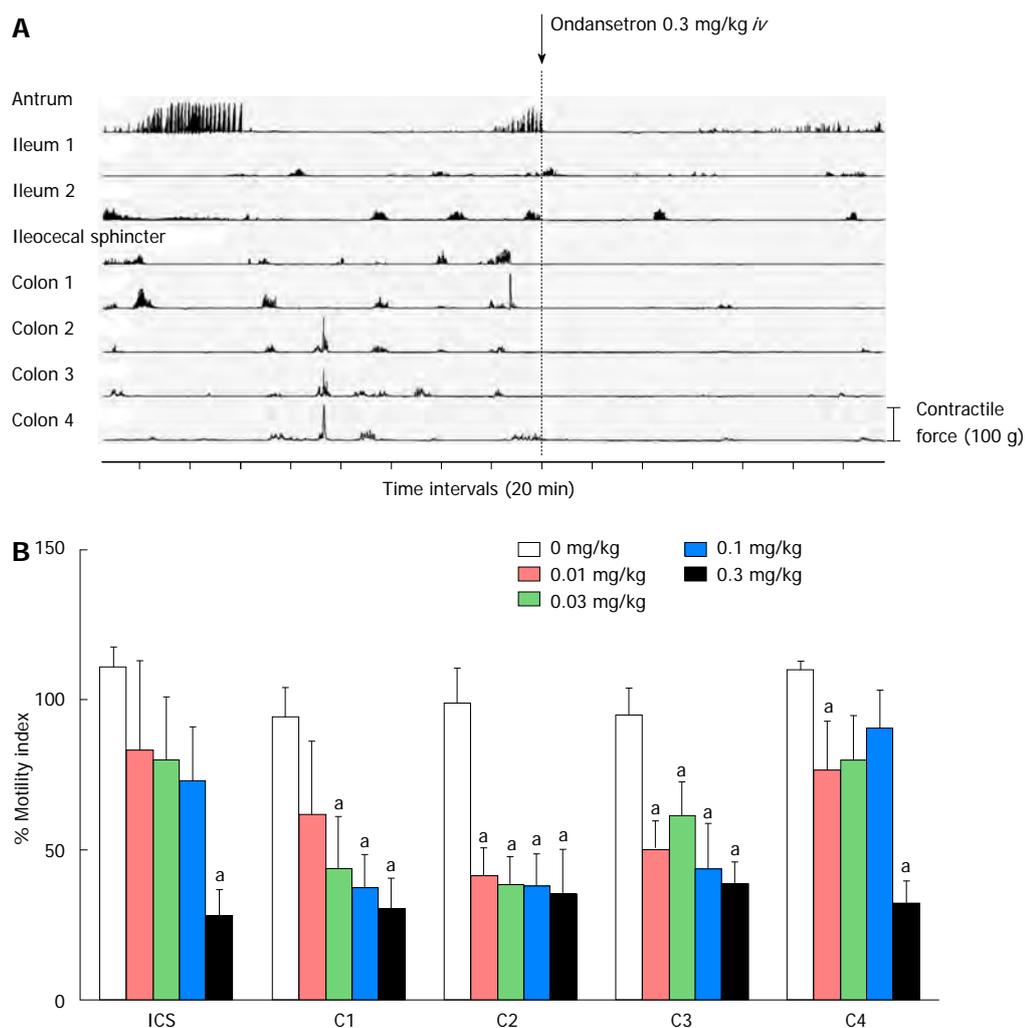


Figure 4 5-hydroxytryptamine 3 receptor antagonists on normal colonic motor activity. A: Typical effect of the 5-hydroxytryptamine (5-HT) 3 receptor antagonist. The 5-HT3 receptor antagonist inhibited phase III of the interdigestive motor complex at the antrum and whole colonic motor activity; B: The inhibition ratio compared with before 5-HT3 receptor antagonist administration. The inhibitory effect of the 5-HT3 receptor antagonist was dose dependent in the proximal colon (ICS-C2). At a dose of 0.3 mg/kg, whole colonic motor activity was inhibited significantly. Values are mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  vs control (0 mg/kg). ICS: ileocecal sphincter; C1-4: Colon 1-4.

## DISCUSSION

Previous studies have investigated the effects of 5-HT receptor antagonists on abnormal colonic motor activity, following either the administration of 5-HT or the induction of stress<sup>[14,26,31,36,37]</sup>. This study is the first to investigate the effects of 5-HT receptor antagonists on normal colonic motor activity in dogs using a force transducer.

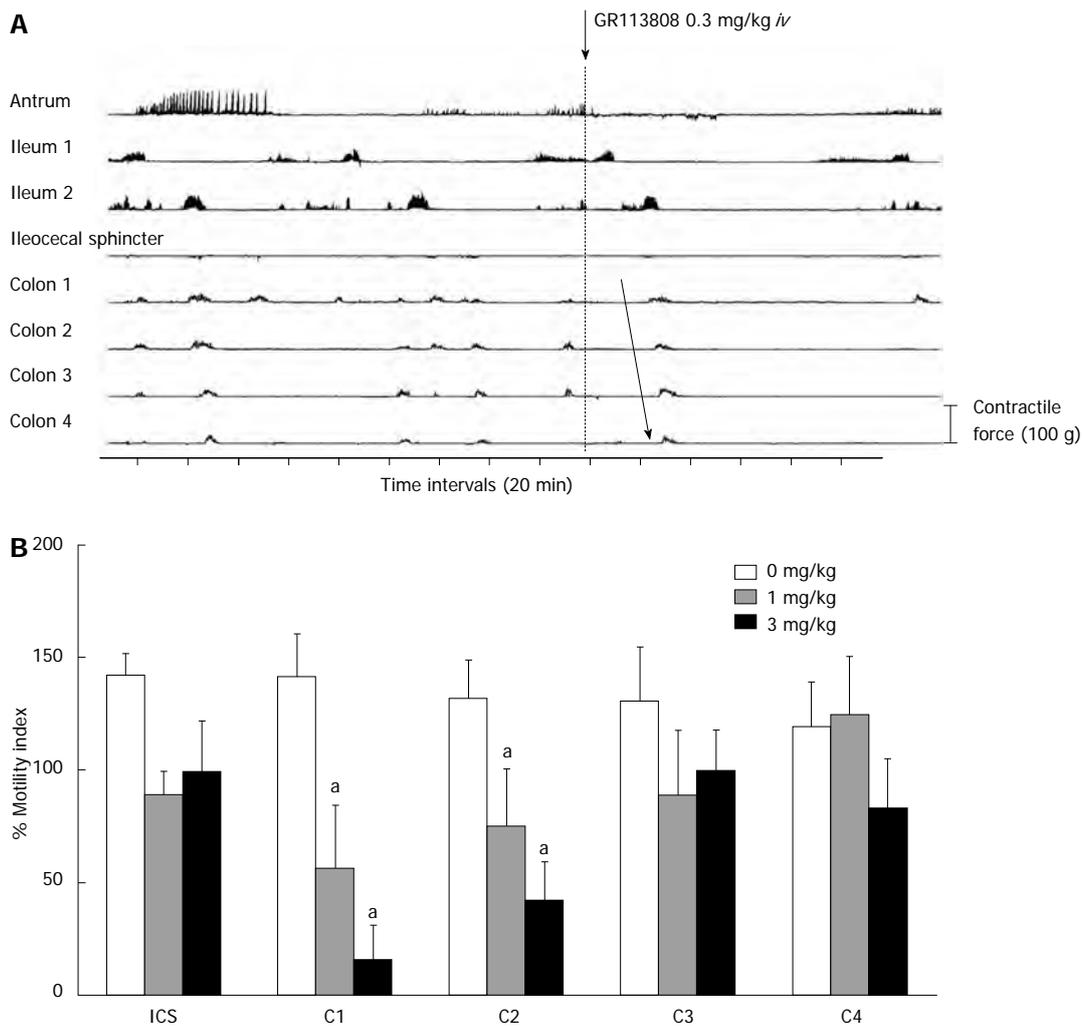
In this study, 5-HT3 and 5-HT4 receptor antagonists inhibited normal colonic motor activity. In contrast, a 5-HT2B receptor antagonist had no contractile effect on normal colonic motor activity. The 5-HT3 receptor antagonist inhibited phase III of the interdigestive motor complex of the antrum. None of the three 5-HT receptor antagonists had contractile effects on the fasted dog terminal ileum.

The effects of 5-HT3 receptor antagonists on colonic motor activity are controversial. In the present study, the 5-HT3 receptor antagonist inhibited normal colonic motor activity. However, Yoshida *et al*<sup>[17]</sup> reported that

ondansetron (GR38032F) had no contractile effect on normal colonic motor activity in the interdigestive state in dogs. In their study, the 5-HT3 receptor antagonist was administered at a high dose of 1 mg/kg. It is possible that a higher dose of the 5-HT3 receptor antagonist may paradoxically have no contractile effect on normal colonic motor activity.

Because we administered the 5-HT3 receptor antagonist systemically, we were unable to determine its mechanism of action. We did observe, however, that the inhibitory effects were exerted in the extrinsic denervation region<sup>[38]</sup> (data not shown), suggesting that it acted locally.

How colonic contractions are generated is unclear. It is known that the pacemaker of colonic contractions lies within the colon wall itself because contractions can occur in an isolated colon. The pacemaker itself, however, has not been characterized. Heredia *et al*<sup>[39]</sup> showed that the initiation of colonic contractions requires serotonin. Dickson *et al*<sup>[40]</sup> demonstrated that 5-HT initially excites



**Figure 5** 5-hydroxytryptamine 4 receptor antagonists on normal colonic motor activity. **A:** Typical effect of the 5-hydroxytryptamine (5-HT) 4 receptor antagonist. The 5-HT<sub>4</sub> receptor antagonist inhibited phase III of the interdigestive motor complex at the antrum and whole colonic motor activity, CMMCs corresponding to phase III of the migrating motor complex at the ileum were sometimes observed (see arrow); **B:** The inhibition ratio compared with before 5-HT<sub>4</sub> receptor antagonist administration. The inhibitory effect of the 5-HT<sub>4</sub> receptor antagonist was dose dependent in the proximal colon (C1-C2). Values are mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  vs control (0 mg/kg). ICS: Ileocecal sphincter; C1-4: Colon 1-4.

5-HT<sub>3</sub> receptors on the mucosal endings of Dogiel type II/AH neurons and that this is coincidental with colonic contractions. It is possible that the inhibitory effects of 5-HT<sub>3</sub> receptor antagonists demonstrated in this study may act at this site. Bharucha *et al.*<sup>[41]</sup> showed that a 5-HT<sub>4</sub> receptor antagonist (SB-207266) tended to delay colonic transit. Consistent with this, in our study, the 5-HT<sub>4</sub> receptor antagonist inhibited colonic motor activity. Studies have shown that receptors on contractile nerves may be more sensitive to receptor antagonism than those on relaxation nerves<sup>[22-25]</sup>. We hypothesize that the 5-HT<sub>4</sub> receptor antagonist inhibitory effect on colonic contractile nerves may be a mechanism that results in the delay of colonic transit.

Glucagon and scopolamine butylbromide are commonly used as antispasmodic premedications for colonoscopy. In addition, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists may also be used as premedications for colonoscopy. In this study, we used ondansetron as the 5-HT<sub>3</sub> receptor antagonist. Ondansetron has no effect on visceral sensa-

tions<sup>[28]</sup>. Visceral hypersensitivity has been proposed as a mechanism of IBS, and Kim *et al.*<sup>[42]</sup> have reported that the degree of pain perception during colonoscopy is higher in IBS patients than in non-IBS patients. Alosetron, which can abolish visceral sensations<sup>[14]</sup>, may be used as a premedication for colonoscopy without pain. However, whether alosetron is effective in controlling colonic motor activity has not been conclusively determined.

In a study by Ohashi-Doi *et al.*<sup>[26]</sup>, a 5-HT<sub>2B</sub> receptor antagonist inhibited visceral hypersensitivity and reduced restraint stress-induced defecation. It is therefore logical to hypothesize that a 5-HT<sub>2B</sub> receptor antagonist may have therapeutic potential for the treatment of non-constipation IBS. Bassil *et al.*<sup>[43]</sup> have previously shown that high-dose (10 and 30 mg/kg) 5-HT<sub>2B</sub> antagonists inhibit colonic motility and defecation in normal mice. The present study did not confirm their findings, as the 5-HT<sub>2B</sub> receptor antagonist had no contractile effect on normal colonic motor activity. However, we only evaluated doses up to 3 mg/kg. By contrast, our previous study showed

that RQ-00310941 at 3 mg/kg *p.o.* inhibited restraint stress-induced defecation in TNBS-treated rats<sup>[34]</sup>. In that preliminary study, rats were administered 3 mg/kg TNBS orally, but the plasma concentration of RQ-00310941 was greater than that in the dogs in the current study, which were also administered 3 mg/kg *iv* (data not shown). We therefore speculate that RQ-00310941 can inhibit restraint stress-induced defecation at higher doses.

In the proximal colon, the inhibitory effects of 5-HT<sub>3</sub> and 5-HT<sub>4</sub> antagonists were dose dependent. However, this inhibition was dose independent in the distal colon. Nagakura *et al.*<sup>[36]</sup> also showed that the effect of a 5-HT<sub>4</sub> receptor agonist on colonic motor activity in the proximal colon was stronger than that in the distal colon. A different distribution of 5-HT receptors in the distal colon compared with the proximal colon may explain this effect. These confounding effects may also be based on a differential sensitivity to drugs in the proximal and distal colon<sup>[44,45]</sup>. Further studies are needed to clarify this result. In conclusion, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists inhibited phase III of the interdigestive motor complex at the antrum and colonic motor activity. The 5-HT<sub>2B</sub> receptor antagonist had no contractile effect on normal colonic motor activity.

## ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Dr. Zen Ito and Dr. Yuichi Tabe. We thank Mr. Arimitsu Bettou for making the jackets for the dogs.

## COMMENTS

### Background

Most of the body's 5-hydroxytryptamine (5-HT) is found in the intestinal tract, where it helps regulate intestinal motor activity. The 5-HT receptors that are known to affect gut motor functions are those belonging to the 5-HT<sub>1</sub>, 2, 3, 4 and 7 subtypes.

### Research frontiers

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder associated with altered motility, secretion and visceral sensation. Recently, 5-HT was identified as the cause of IBS.

### Innovations and breakthroughs

Previous studies have investigated the effects of 5-HT receptor antagonists on abnormal colonic motor activity, following either the administration of 5-HT or the induction of stress. This study is the first to investigate the effects of 5-HT receptor antagonists on normal colonic motor activity in dogs.

### Applications

The study results suggest that 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists may also be used as premedications for colonoscopy. 5-HT<sub>2B</sub> receptor antagonists may be used for the treatment of diarrhea-predominant IBS without the side effect of constipation.

### Terminology

Motility index (MI): MI was defined as the integrated area between baseline (zero level) and the contractile wave expressed in motor units. MI is an index used when analyzing gastrointestinal motility. 5-HT: Over 95% of the 5-HT in the body is found in the gastrointestinal tract; 90% of the gastrointestinal 5-HT is found within enterochromaffin cells. 5-HT receptors are known to affect gut motor functions.

### Peer review

This is a good descriptive study in which the authors analyze the effects of 5-HT<sub>3</sub>, 5-HT<sub>4</sub> and 5-HT<sub>2B</sub> receptor antagonists on normal gastrointestinal mo-

tility. 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptor antagonists inhibited colonic motor activity. The 5-HT<sub>2B</sub> receptor antagonist had no contractile effect on normal colonic motor activity.

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## Colonic diverticulitis with comorbid diseases may require elective colectomy

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### Abstract

**AIM:** To investigate the comorbid disease could be the predictors for the elective colectomy in colonic diverticulitis.

**METHODS:** A retrospective chart review of 246 patients with colonic diverticulitis admitted between 2000 and 2008 was conducted, and 19 patients received emergent operation were identified and analyzed. Data were collected with regard to age, sex, albumin level on admission, left or right inflammation site, the history of recurrent diverticulitis, preoperative comorbidity, smoking habits, medication, treatment policy, morbidity, and mortality. Preoperative comorbid diseases included cardiovascular disease, diabetes, pulmonary disease, peptic ulcer disease, gouty arthritis, and uremia. Medications in use included non-steroidal anti-inflammatory drugs, acetylsalicylic acid (Aspirin), and corticosteroids. Univariate and multivariate logistic regression analyses

were performed to identify the relevant risk factors correlating to colectomy.

**RESULTS:** The mean age of the 246 patients was 69.5 years (range, 24-94 years). Most diverticulitis could be managed with conservative treatment ( $n = 227$ , 92.3%), and urgent colectomy was performed in 19 patients (7.7%). There were three deaths in the surgical group and four deaths in the nonsurgical group. The overall mortality rate in the study was 1.7% among patients with conservative treatment and 15.7% among patients undergoing urgent colectomy. Multiple logistic regression analysis indicated that comorbidities were risk factors for urgent colectomy for diverticulitis.

**CONCLUSION:** To avoid high mortality and morbidity related to urgent colectomy, we suggest that patients with colonic diverticulitis and comorbid diseases may require elective colectomy.

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**Key words:** Colonic diverticulitis; Colectomy; Comorbid disease

**Core tip:** Colonic diverticulitis can usually be managed with conservative treatment. However, in select groups of patients with recurrent persistent infection causing life-threatening septic shock, emergent and risky surgical management may be necessary. Elective colectomy for diverticulitis has been discussed in many reports. However, the criteria for elective surgery for colonic diverticulitis still remain controversial. Our data indicate that diverticulitis with comorbid disease increases the operative risk in urgent surgery. Therefore, if a patient has diverticulitis with comorbid disease, an elective colectomy may prevent the consequences of an urgent operation.

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Colonic diverticulitis with comorbid diseases may require elective colectomy. *World J Gastroenterol* 2013; 19(39): 6613-6617 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6613.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6613>

## INTRODUCTION

Diverticular disease of the colon has an overall prevalence of less than 10% in individuals under 40 years old and an estimated 50%-66% prevalence in individuals 80 years old or older<sup>[1-4]</sup>. A diverticulum is the herniation of the mucosa and submucosa through the muscular layers of the colon and occurs because of increased intraluminal pressure and weakness in segments of the colonic wall<sup>[5]</sup>. Overall, 10%-25% of patients with diverticular disease will experience diverticulitis and its complications<sup>[6,7]</sup>. Diverticulitis is the inflammation of a diverticulum or diverticula and can be classified as uncomplicated or complicated. Cases involving abscesses, perforation, obstruction, fistula formation or peritonitis are defined as complicated diverticulitis<sup>[8]</sup>. They may require percutaneous drainage of an abscess or emergent surgery. On the other hand, elective colectomy is recommended if an episode of complicated diverticulitis was treated non-operatively<sup>[5,6]</sup>. Elective colectomy to prevent recurrent attacks of diverticulitis or emergent surgery was previously advised<sup>[6]</sup>. However, newer recommendations have called for a revision of the practice of aggressive surgical resection in patients with uncomplicated disease, despite recurrence or age<sup>[8]</sup>. The basis of these arguments is that a majority of patients do not appear to progress from uncomplicated to complicated disease over time<sup>[9,10]</sup>. Moreover, recurrent diverticulitis has not been demonstrated to be a risk for emergency surgery<sup>[8,9]</sup>. The aim of this study was to identify which comorbid diseases could be risk factors for urgent colectomy to aid in the management of colonic diverticulitis. Using this information, we can identify patients who may benefit from early elective colectomy.

## MATERIALS AND METHODS

The charts of 246 patients who had been admitted to the Tri-Service General Hospital, Taipei, Taiwan, between 2000 and 2008 with a diagnosis of diverticulitis were reviewed, and 19 patients who received emergent operations were identified and analyzed retrospectively.

Data were collected with regard to age, sex, albumin level on admission, left or right inflammation site, history of recurrent diverticulitis, preoperative comorbidity, smoking habits, medication, treatment policy, morbidity, and mortality. The diagnosis of colonic diverticulitis was based on the findings of computed tomography, which has a high diagnostic sensitivity of approximately Ninety-three percent to ninety-seven percent and a specificity of

approximately 100%<sup>[4]</sup>. Urgent colectomy was indicated in patients with peritonitis that could not be corrected by medical treatment or drainage. Preoperative comorbid diseases included cardiovascular disease (*e.g.*, hypertensive cardiovascular disease and coronary artery disease), diabetes, pulmonary disease (*e.g.*, chronic obstructive pulmonary disease and asthma), peptic ulcer disease (*e.g.*, gastric ulcer and duodenal ulcer), gouty arthritis, and uremia. Medications in use included non-steroidal anti-inflammatory drugs (NSAIDs), acetylsalicylic acid (aspirin), and corticosteroids.

The mean values were compared using an independent *t* test, and  $\chi^2$  or Fisher's exact test was used to compare proportions. A value of  $P < 0.05$  was considered statistically significant. Data were analyzed using SPSS version 15.0 (SPSS Inc., Chicago, IL, United States) for Windows.

## RESULTS

In total, 246 patients were admitted with a diagnosis of colonic diverticulitis. The mean age was 69.5 years (range, 24-94 years); 144 of the 246 patients were men (58.5%), and 102 were women (41.5%). Most episodes could be managed with conservative treatment consisting of antibiotics and bowel rest ( $n = 227$ , 92.3%), but urgent colectomy was performed in 19 patients (7.7%) (Table 1). All of these patients received Hartmann's operation. However, there were three deaths in the surgical group, two from postoperative lobar pneumonia complicated with septic shock and acute respiratory distress syndrome and the other from urinary tract infection. There were four deaths in the non-surgical group. Two deaths (ages 81 and 79 years; the patient's family refused surgical intervention in both cases) in the nonsurgical group were the result of uncontrolled intra-abdominal infection complicated with septic shock during hospitalization. The other two deaths were due to lobar pneumonia (Table 2). The overall mortality rate was 2.8% ( $n = 7$ ), and it was 15.7% ( $n = 3$ ) in the group that underwent urgent colectomy. Ten patients had wound infections after colectomy, and four were returned to the operating room for surgical debridement. The morbidity rate in the urgent surgery group was 52.6% ( $n = 10$ ) (Table 3).

Univariate analysis with logistic regression indicated that the factors associated with a greater likelihood of having an urgent colectomy were albumin level on admission (OR = 13.488,  $P < 0.001$ ), comorbidity with cardiovascular disease (OR = 3.203,  $P = 0.019$ ), type 2 diabetes (OR = 3.311,  $P = 0.019$ ) or gouty arthritis (OR = 9.777,  $P < 0.001$ ), and use of anticoagulants (OR = 3.200,  $P = 0.023$ ) or NSAIDs (OR = 9.603,  $P < 0.001$ ; Table 4). According to the multivariate analysis with logistic regression, only the albumin level, comorbidity with type 2 diabetes, the use of NSAIDs, and smoking remained risk factors for urgent colectomy for colonic diverticular disease (Table 5).

**Table 1** Characteristics of patients with colonic diverticulitis *n* (%)

	Surgery group ( <i>n</i> = 19)	Non-surgery group ( <i>n</i> = 227)	<i>P</i> value
Age (yr)	66.21 ± 15.77	69.81 ± 12.36	0.344
Sex			0.670
Male	12 (36.6)	132 (58.1)	
Female	7 (63.2)	95 (41.9)	
Smoking			0.005
Yes	6 (31.6)	23 (10.1)	
No	13 (68.4)	204 (89.9)	
Albumin			< 0.001
< 3	11 (57.9)	21 (9.3)	
≥ 3	8 (42.1)	206 (90.7)	
Aspirin			0.017
Yes	7 (36.8)	35 (15.4)	
No	12 (63.2)	192 (84.6)	
NSAIDs			< 0.001
Yes	7 (36.8)	13 (5.7)	
No	12 (63.2)	214 (94.3)	
Steroids			0.138
Yes	2 (10.5)	8 (3.5)	
No	17 (89.5)	219 (96.5)	
Cardiovascular disease			0.014
Yes	8 (42.1)	42 (18.5)	
No	11 (57.9)	185 (81.5)	
DM			0.014
Yes	7 (36.8)	34 (15.0)	
No	12 (63.2)	193 (85.0)	
Liver disease			0.862
Yes	1 (5.3)	10 (4.4)	
No	18 (94.7)	217 (95.6)	
Uremia			0.997
Yes	1 (5.3)	12 (5.3)	
No	18 (94.7)	215 (94.7)	
Gout			< 0.001
Yes	5 (26.3)	8 (3.5)	
No	14 (73.7)	219 (96.5)	
Pulmonary disease			0.658
Yes	2 (10.5)	18 (7.9)	
No	17 (89.5)	209 (92.1)	
Peptic ulcer disease			0.694
Yes	2 (10.5)	21 (9.3)	
No	17 (89.5)	206 (90.7)	

DM: Diabetes mellitus; NSAID: Non-steroidal anti-inflammatory drug.

## DISCUSSION

Emergent surgery should be considered for diverticulitis when the patient has peritonitis from perforation or obstruction causing unstable vital signs, when the patient fails to respond to conservative treatment within the first week of hospitalization, and when the patient presents with a large abscess (> 5 cm) that is undrainable by interventional radiology<sup>[11]</sup>. Approximately 5.5% of patients who recover from an initial episode of diverticulitis require emergency surgical intervention<sup>[12]</sup>. Although the mortality and morbidity rates are low, in a previous study, as in our study, they are higher when an urgent operation was performed. The mortality and morbidity rates were high in our operated group (15.7% and 52.6%, respectively). These results are in agreement with previous reports that indicated that patients who require surgical intervention have high mortality and morbidity rates of

**Table 2** Characteristics of mortality case

Case	Age (yr)	Sex	Risk factors
Surgery			
1	75	Male	Gout, low albumin level, NSAIDs
2	60	Male	Uremia, DM
3	77	Female	Pulmonary disease, low albumin level
Non-surgery			
1	81	Male	DM, uremia
2	79	Male	NSAIDs, low albumin level, uremia
3	68	Male	Pulmonary disease, DM
4	74	Female	Pulmonary disease, uremia

DM: Diabetes mellitus; NSAID: Non-steroidal anti-inflammatory drug.

**Table 3** Surgical complication of urgent surgery *n* (%)

Complications	<i>n</i> (%)
Wound infection	10 (52.6)
Wound infection needing debridement	4 (21.0)
Pneumonia complicated with acute respiratory distress syndrome	2 (10.5)
Urinary tract infection	1 (5.2)
Mortality	3 (15.7)

**Table 4** Risk factors for urgent colectomy for colonic diverticulitis according to univariate analysis

Variables	B	SE	OR (95%CI)	<i>P</i> value
Smoking	1.409	0.540	4.094 (1.420-11.805)	0.009
Albumin	2.602	0.518	13.488 (4.886-37.232)	< 0.001
Aspirin	1.163	0.510	3.200 (1.178-8.693)	0.023
NSAIDs	2.262	0.555	9.603 (3.237-28.485)	< 0.001
CV disease	1.164	0.495	3.203 (1.214-8.454)	0.019
DM	1.197	0.511	3.311 (1.217-9.009)	0.019
Gout	2.280	0.633	9.777 (2.826-33.823)	< 0.001

DM: Diabetes mellitus; NSAID: Non-steroidal anti-inflammatory drug; CV: Cardiovascular.

approximately 4.5%-16.7% and 27.2%-71.1%, respectively<sup>[1-3]</sup>. Hartmann's operation is a potential choice for the surgical intervention if the patient has diverticulitis with necrotic perforation. However, laparoscopic washout with diversion may be used if the patient has diverticulitis without necrotic perforation. In our study, all patients were subjected to Hartmann's procedure because they had diverticulitis with necrotic perforation.

To prevent a delay in treatment or consequent resource waste, elective surgery for patients with comorbidities may be recommended. In the previous practice parameters for sigmoid diverticulitis, elective surgery was recommended after two episodes of diverticulitis<sup>[6]</sup>. Richards *et al*<sup>[13]</sup> demonstrated that prophylactic colectomy is associated with increased life expectancy and quality-of-life years when performed after the third attack. However, Ricciardi *et al*<sup>[14]</sup> found that the decline in the surgical treatment for diverticulitis is not associated with an increase in complicated diverticulitis, which indicates that elective surgery has a minimal effect on preventing

**Table 5 Risk factors for urgent colectomy for colonic diverticulitis according to multivariable analysis**

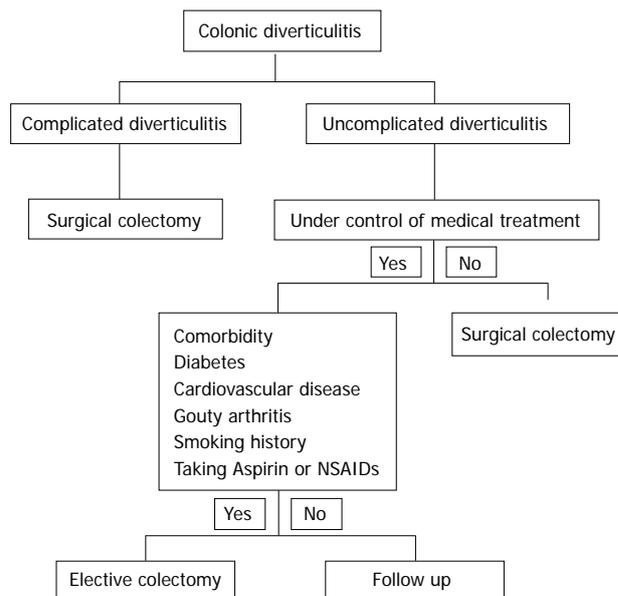
Variables	B	SE	OR (95%CI)	P value
Age	-0.046	0.662	0.955 (0.261-3.497)	0.944
Sex	0.001	0.025	1.001 (0.953-1.050)	0.983
Smoking	2.169	0.873	8.747 (1.580-48.405)	0.013
Albumin	3.467	0.806	32.049 (6.599-155.644)	< 0.001
Aspirin	-0.873	1.382	0.418 (0.028-6.270)	0.527
NSAIDs	3.323	1.212	27.745 (2.578-298.594)	0.006
Steroids	0.785	1.313	2.193 (0.167-28.780)	0.550
CV disease	1.320	1.262	3.745 (0.316-44.390)	0.295
DM	1.810	0.726	6.109 (1.473-25.333)	0.013
Liver disease	-0.291	1.373	0.748 (0.051-11.028)	0.832
Uremia	0.898	1.250	2.454 (0.212-28.416)	0.473
Gout	0.470	1.335	1.600 (0.117-21.911)	0.725

DM: Diabetes mellitus; NSAID: Non-steroidal anti-inflammatory drug; CV: Cardiovascular.

emergency colectomies. The recent recommendation of the American Society of Colon and Rectum Surgeons stated that “the decision to recommend elective sigmoid colectomy after recovery from acute diverticulitis should be made on a case-by-case basis”<sup>[15]</sup>. The decision should be made based on the age and the medical condition of the patients, the frequency and severity of the attacks, whether there are persistent symptoms and the suspicion of the malignancy<sup>[15]</sup>. Furthermore, elective surgery is also recommended if an episode of complicated diverticulitis is treated nonoperatively. The predictors of diverticulitis requiring surgery have not been determined, which is still a vexing problem confronting surgeons.

Our study was a retrospective analysis of 246 patients with colonic diverticulitis treated at a single medical center over 9 years. No difference was found in the distribution of colonic diverticulitis between the sexes ( $P = 0.671$ ), and this trend was the same for cases requiring emergent surgery. The ages of patients with diverticulitis had no significant association with surgery ( $P > 0.344$ ). Patient nutritional status upon admission affected the length of hospital stay for patients with colonic diverticulosis. Serum albumin levels correlated negatively with the length of hospital stay<sup>[16,17]</sup>. Moreover, it is well known that there is a correlation between malnutrition and infection, poor wound healing, and the overgrowth of bacteria in the alimentary tract. In the present study, 57.9% of colonic diverticulitis patients in the emergent surgery group had albumin levels below 3 g/dL, whereas this was true of only 9.3% of patients in the non-surgery group. All patients with low albumin (< 3 g/dL) levels had parenteral nutrition.

Among the comorbidities, type 2 diabetes mellitus independently increased the severity of diverticulitis ( $P = 0.019$ ). It is well established that diabetes mellitus has a negative effect on immunity. Polymorphonuclear leukocyte function, leukocyte adherence, chemotaxis, and phagocytosis are all affected in patients with diabetes mellitus<sup>[18]</sup>. Gouty arthritis have also been demonstrated to be a risk factor of urgent operation ( $P < 0.001$ ). How-



**Figure 1 Management of patients with colonic diverticulitis.** NSAIDs: Non-steroidal anti-inflammatory drugs.

ever, this relation may be because of the medication used in treatment because patients with these diseases take NSAIDs for pain control. It is known that there is a correlation between NSAIDs and perforation of colonic diverticula. Several reports have indicated that patients with perforated diverticular disease are significantly more likely to be taking NSAIDs than patients without disease<sup>[19-21]</sup>. The inhibition of cyclo-oxygenase 1 (COX-1) results in deficient levels of prostaglandins, which are related to the protection of bowel mucosa. On the other hand, COX-2 inhibition leads to the failure of the immune response to localize a microperforation. In these patients, continuation of their non-steroidal anti-inflammatory treatments is highly likely to impair colonic mucosal repair<sup>[19-21]</sup>. Cardiovascular disease has also been shown to be associated with urgent colectomy in diverticulitis. In the present study, all of the patients with cardiovascular diseases were taking acetylsalicylic acid, known as aspirin, as an anti-coagulant for preventive treatment. Aspirin is also classified as an NSAID because of its inhibition of platelet COX activity and is considered to predict colonic perforation<sup>[22]</sup>. Smoking was one of the independent factors associated with emergent operation. Smoking may enhance or precipitate the inflammatory process in diverticulitis. Nicotine in cigarettes is a smooth muscle relaxant, which may counteract the colonic muscle spasm that correlates with the pathogenesis of diverticular disease<sup>[23]</sup>. Nicotine may also decrease mucosal immunity by inhibiting the synthesis of proinflammatory cytokines such as interleukin  $\alpha$  and tumor necrosis factor  $\alpha$  in colonic mucosa. Smoking could also cause systemic sepsis via the promotion of oxidative damage by generating oxygen free radicals<sup>[23]</sup>. Steroid treatment also has a negative effect on mucosal regenerative activity<sup>[24]</sup>. In our study, it did not significantly predict diverticular perforation. This

is most likely because the number of patients with steroid treatment in our series was small.

Our results indicated several comorbid diseases are risk factors to urgent colectomy. There were 7.7% of patients with colonic diverticular disease who had to receive urgent colectomy despite conservative treatment including antibiotics and bowel rest. Preoperative comorbid diseases may increase the operative risk in urgent surgery, and the outcome is poor. The mortality and morbidity rates are up to 15.7% and 52.6%, respectively. To avoid high mortality and morbidity related to urgent colectomy. We suggested that patients with colonic diverticulitis and comorbid disease may require elective colectomy (Figure 1).

## COMMENTS

### Background

Colonic diverticulitis with comorbid disease may get fatal complication after urgent surgery. Therefore, To avoid high mortality and morbidity related to urgent colectomy, the authors suggest that patients with colonic diverticulitis and comorbid diseases may require elective colectomy.

### Research frontiers

This study is designed to clarify the predictors of elective colectomy for colonic diverticulitis.

### Innovations and breakthroughs

Comorbid disease could be the predictors of elective colectomy for colonic diverticulitis.

### Applications

Provide a possible policy of surgical management for the patients with colonic diverticulitis.

### Peer review

This is an interesting paper stressing the importance of comorbidities in the treatment of diverticulitis.

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## Anisodamine accelerates spontaneous passage of single symptomatic bile duct stones $\leq 10$ mm

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group received the same volume of 0.9% isotonic saline for 2 wk. Patients underwent imaging studies and liver-function tests every week for 4 wk. The rate of spontaneous passage of CBD stones was analyzed.

**RESULTS:** The rate of spontaneous passage of CBD stones was significantly higher in the anisodamine group than that in the control group (47.0% vs 22.7%). Most (87.2%, 41/47) stone passages in the anisodamine group occurred in the first 2 wk, and passages in the control group occurred at a comparable rate each week. Factors significantly increasing the possibility of spontaneous passage by univariate logistic regression analyses were stone diameter ( $< 5$  mm vs  $\geq 5$  mm and  $\leq 10$  mm) and anisodamine therapy. Multivariate logistic regression analyses revealed that these two factors were significantly associated with spontaneous passage.

**CONCLUSION:** Two weeks of anisodamine administration can safely accelerate spontaneous passage of single and symptomatic CBD stones  $\leq 10$  mm in diameter, especially for stones  $< 5$  mm.

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### Abstract

**AIM:** To investigate the rate of spontaneous passage of single and symptomatic common bile duct (CBD) stones  $\leq 10$  mm in diameter in 4 wk with or without a 2-wk course of anisodamine.

**METHODS:** A multicenter, randomized, placebo-controlled trial was undertaken. A total of 197 patients who met the inclusion criteria were enrolled. Ninety-seven patients were assigned randomly to the control group and the other 100 to the anisodamine group. The anisodamine group received intravenous infusions of anisodamine (10 mg every 8 h) for 2 wk. The control

**Key words:** Common bile duct stones; Anisodamine; Spontaneous passage; Success rate; Randomized controlled trial

**Core tip:** Common bile duct (CBD) stones are known to pass spontaneously in many patients. This phenomenon has not been given sufficient emphasis in terms of optimizing the timing of management of CBD stones. We investigated the rate of spontaneous passage of single and symptomatic CBD stones  $\leq 10$  mm in diameter in 4 wk with or without a 2-wk course of anisodamine in a multicenter, randomized, placebo-controlled trial. Anisodamine administration for 2 wk can safely accelerate spontaneous passage of single and symp-

tomatic CBD stones  $\leq$  10 mm in diameter, especially for stones < 5 mm.

Gao J, Ding XM, Ke S, Zhou YM, Qian XJ, Ma RL, Ning CM, Xin ZH, Sun WB. Anisodamine accelerates spontaneous passage of single symptomatic bile duct stones  $\leq$  10 mm. *World J Gastroenterol* 2013; 19(39): 6618-6624 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6618.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6618>

## INTRODUCTION

Gallstone disease is one of the most common digestive diseases worldwide. Common bile duct (CBD) stones may develop in 3%-14.7% of patients who undergo cholecystectomy<sup>[1-4]</sup>. The stones can present in various ways or may be asymptomatic. Common symptoms of CBD stones include biliary colic, jaundice, acute pancreatitis, acute cholangitis, or a combination of these symptoms<sup>[4]</sup>.

Management of symptomatic CBD stones has evolved over the recent two decades and continues to evolve<sup>[5,6]</sup>. Before the advent of laparoscopy, patients with CBD stones requiring surgical treatment usually underwent laparotomy with CBD exploration and T-tube placement. Alternatively, they underwent endoscopic retrograde cholangiopancreatography (ERCP)/endoscopic sphincterotomy (EST) by endoscopists<sup>[7-9]</sup> to avoid surgical CBD exploration. In recent years, laparoscopic CBD exploration has emerged as an alternative to ERCP/EST and a successor to open CBD exploration for the management of CBD stones<sup>[10-12]</sup>.

The management strategy of CBD stones has focused mainly on the choice of treatment rather than timing. A consensus on the timing of management of symptomatic CBD stones is lacking. Most physicians, surgeons and endoscopists propose that symptomatic CBD stones should be extracted promptly<sup>[5]</sup>. However, accumulating evidence has shown that CBD stones often pass spontaneously, which raises the possibility of avoiding CBD intervention altogether<sup>[1,13-16]</sup>. The reported prevalence of spontaneous passage varies considerably, mainly because of different inclusion criteria and research methods used<sup>[1,13-15]</sup>, but authors conclude that a significant portion of CBD stones may pass spontaneously with conservative treatment and do not need to be extracted by surgical or endoscopic means.

Spontaneous passage of CBD stones is dependent upon relaxation of the sphincter of Oddi. Hence, it is logical to use a medication to relax the sphincter of Oddi to accelerate the passage of CBD stones. Anisodamine, 6(s)-hydroxyhyoscyamine, is a belladonna alkaloid derived from the Chinese medicinal herb *Scopolia tangutica* Maxim of the Solanaceae family. It appears to be a non-selective M-cholinergic antagonist presumably capable of blockade at all M-choline receptor subtypes<sup>[17-23]</sup>. Little clinical information is available regarding its toxicity in humans.

Based upon animal studies, it is less toxic than atropine or scopolamine<sup>[18]</sup>. Since the late 1970s in China, anisodamine has been employed almost exclusively as an OTC anti-spasmodic agent for relieving colic pain in the treatment of CBD stones<sup>[17]</sup>.

We first attempted to use anisodamine to aid spontaneous passage of symptomatic CBD stones by prolonging its use to 2 wk in 2007, and noticed that it might accelerate their spontaneous passage. In 2009, we initiated a prospective, randomized, placebo-controlled trial to observe the rate of spontaneous passage of single and symptomatic CBD stones  $\leq$  10 mm diameter in 4 wk with or without a 2-wk course of anisodamine.

## MATERIALS AND METHODS

### Design

This study was a multicenter, randomized, placebo-controlled trial comparing the rate of spontaneous passage of single and symptomatic CBD stones  $\leq$  10 mm in diameter in 4 wk with or without a 2-wk course of anisodamine. The following hospitals in China participated in the study: Beijing Chaoyang Hospital affiliated to Capital Medical University, Beijing, China; 254 Hospital of PLA, Tianjin, China; Chaoyang Central Hospital, Liaoning, China; and Zhanhua People's Hospital, Shandong, China. Each center obtained approval from the responsible ethics committee according to the standards of the Declaration of Helsinki before the trial according to the current practice and guidelines in China. The first or primary approval of the study was obtained at the Beijing Chaoyang Hospital affiliated to Capital Medical University. All research personnel and collaborators were trained accordingly.

All patients with CBD stones were informed of the currently available therapies to extract stones from the bile duct: ERCP/EST, laparoscopic surgery, or laparotomy. All patients were informed of the potential morbidity of each endoscopic or surgical procedure. All participants provided written informed consent for inclusion in the study. Patients were recruited into the study between January 2009 and April 2013.

Patients were enrolled if they met the following three inclusion criteria: (1) clinical presentation was consistent with biliary colic, cholangitis, jaundice, or/and acute pancreatitis. Jaundice was defined as a bilirubin level of > 50  $\mu\text{mol/L}$  (normal range, 7-20  $\mu\text{mol/L}$ ). Acute pancreatitis was defined by at least two of the following criteria: characteristic abdominal pain, serum amylase and/or lipase values exceeding three times the upper limit of normal, and a computed tomography (CT) scan demonstrating the characteristic changes of acute pancreatitis<sup>[24]</sup>; (2) abnormal liver function: high serum levels of aminotransferase, alkaline phosphatase, or  $\gamma$ -glutamyl transpeptidase of more than twice the normal values; and (3) a single CBD stone  $\leq$  10 mm in diameter confirmed by conventional CT or magnetic resonance cholangiopancreatography (MRCP).

Exclusion criteria were: (1) histories of emergency ERCP/EST or other procedures for severe cholangitis or pancreatitis; (2) CBD stones > 10 mm (which rarely pass spontaneously); and (3) contraindications to the use of anisodamine, such as glaucoma or cerebral hemorrhage.

Patients were assigned to the control group or anisodamine group by a computerized randomization process, and all patients were blinded to the allocation.

### Treatment protocol

For patients with jaundice or isolated abnormal liver test results, a pharmacological strategy for protection against liver injury was adopted: vitamin E, vitamin C, glutathione and tiopronin. Patients with biliary colic were treated with non-steroidal anti-inflammatory drugs for pain relief<sup>[25]</sup>. Patients with cholangitis were treated with antibiotics. For patients with acute pancreatitis, support measures were adopted: aggressive hydroelectrolytic replacement, analgesia and nutritional support treatment.

Patients in the anisodamine group received intravenous infusions of anisodamine (10 mg every 8 h) for 2 wk in addition to the treatments mentioned above. Anisodamine was terminated within the following 2 wk. Patients in the control group received the same volume of 0.9% isotonic saline for 2 wk in addition to the treatments mentioned above.

Patients who developed severe pancreatitis<sup>[24]</sup>, severe cholangitis<sup>[26]</sup>, or aggravated jaundice during this conservative treatment period dropped out of the study.

### Data collection

All data (demographic characteristics, nature of clinical presentation, imaging reports, and laboratory results) were collected for analyses by two trained physicians. Imaging reports were completed by two radiologists who had > 10 years of experience and were blinded to the allocation.

### Study endpoints

The primary endpoint of the study was the rate of spontaneous passage of CBD stones in 4 wk. The secondary endpoints were the safety of anisodamine and the dropout rate.

All patients underwent monitoring *via* conventional CT or MRCP and liver-function tests at a 1-wk interval for 4 wk. Spontaneous passage of CBD stones was defined as no signs of CBD stones upon CT or MRCP and normal results of liver function tests after conservative treatment.

Patients who experienced successful passage of CBD stones underwent only laparoscopic cholecystectomy (LC) without CBD intervention. However, if they did not experience successful passage, LC and laparoscopic CBD exploration *via* the transcystic or transduction route were performed. Patients with a history of cholecystectomy did not receive further treatment after successful passage of CBD stones. However, if the CBD stones failed to pass spontaneously, the patients underwent ERCP/EST.

### Statistical analysis

Frossard *et al.*<sup>[13]</sup> reported that the rate of spontaneous passage of CBD stones within 1 mo was 21%. We estimated that the rate of spontaneous passage of CBD stones was 20% because the patients and research methods in the present study were similar to those of Frossard *et al.*<sup>[13]</sup>. Furthermore, the number of patients predicted to be necessary for statistical validity was based on the premise of improving the rate of spontaneous passage of CBD stones from 20% to 40%, with alpha set at 0.05 and beta set at 0.2, yielding a power of 80%. A two-tailed test was used. We calculated that 82 patients would be required in each arm of the study for a total projected study population of 164 patients. After taking a dropout rate of 20% into account, 197 patients would be required.

Continuous data are the mean  $\pm$  SD, and an independent *t* test was used to examine differences between the two groups. Categorical variables were presented as number (%) and analyzed using the  $\chi^2$  test or Fisher's exact test. A stepwise logistic regression model was used to determine the effects of multiple factors on spontaneous passage. Significance was accepted at the 5% level. All CIs were reported at the 95% level. All statistical computations were undertaken using Statistical Package for the Social Sciences ver15.0 (SPSS, Chicago, IL, United States).

## RESULTS

Of the 450 patients with CBD stones who were screened at the four participating hospitals of the study between January 2009 and April 2013, 197 met the inclusion criteria and were enrolled (Figure 1). Ninety-one (46.2%) patients were male and 106 patients were female (53.8%). Ninety-seven patients were randomized to the control group, and 100 to the anisodamine group. No difference in baseline patient characteristics was observed between control and anisodamine groups (Table 1). During the period of conservative treatment, 11 (5.6%) patients (4 in the control group and 7 in the anisodamine group) dropped out because of aggravation of cholangitis (8 patients), pancreatitis (1 patient) and jaundice (2 patients). For these patients, laparoscopic cholecystostomy was carried out in 1 patient, percutaneous transhepatic gallbladder drainage in 2 patients, and ERCP/EST in 8 patients. There was no mortality in the 11 patients.

Spontaneous passage of CBD stones occurred in 22.7% (22/97) and 47.0% (47/100) of patients in control and anisodamine groups, respectively ( $P < 0.05$ ) (Figure 2, Table 2). For subgroup patients with stones < 5 mm in diameter, this rate was significantly higher in the anisodamine group than that in the control group (71.7% *vs* 31.8%,  $P < 0.05$ ), and for those with stones of  $\geq 5$  mm and  $\leq 10$  mm, this rate was also significantly higher in the anisodamine group than that in the control group (27.3% *vs* 15.1%,  $P < 0.05$ ) (Table 2). In the control group, the rate of spontaneous passage of CBD stones in each of the 4 wk was not significantly different ( $P >$

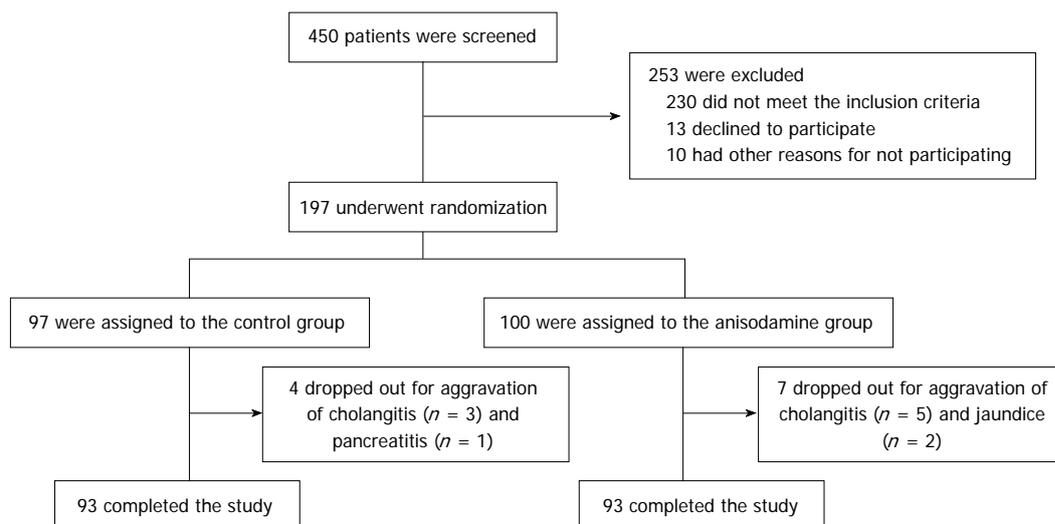


Figure 1 Enrollment and outcomes.

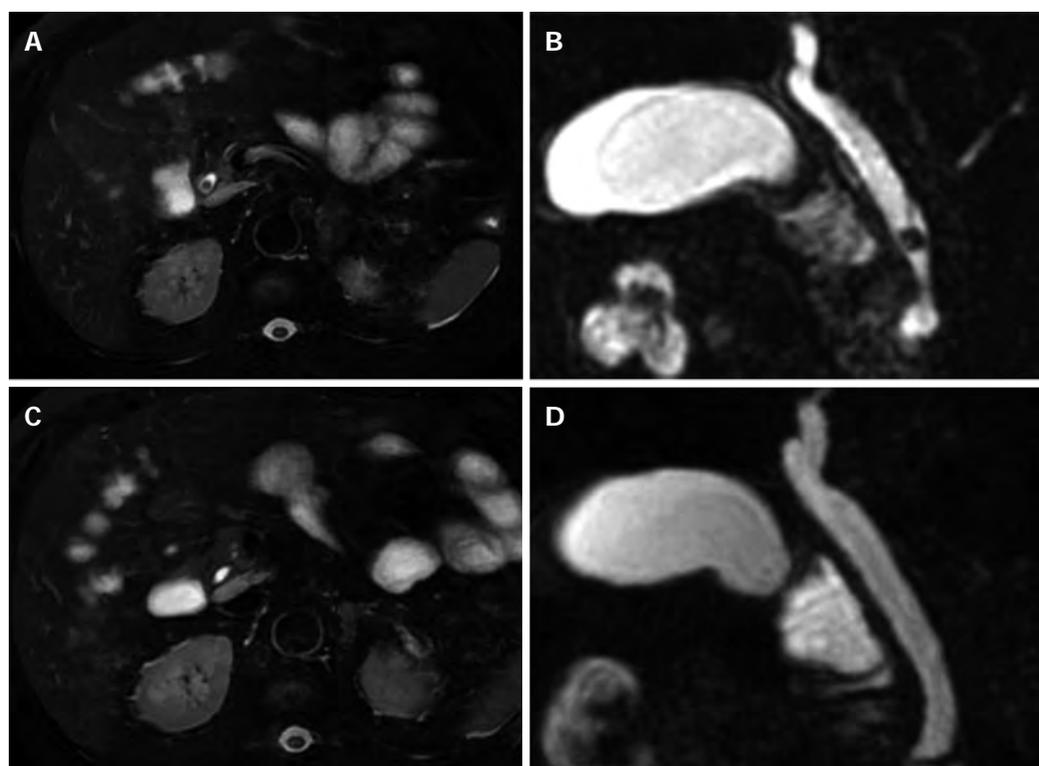


Figure 2 A 60-year-old male with gallstones and a common bile duct stone of 7.0 mm in diameter received anisodamine for 2 wk. The common bile duct (CBD) stone passed spontaneously within 2 wk after treatment. A: Magnetic resonance imaging (MRI) on cross-sectional T2-weighted imaging showing a filling defect in the CBD; B: Magnetic resonance cholangiopancreatography (MRCP) showing a 7-mm filling defect in the dilated CBD; C: Repeat MRI in the second week after conservative treatment illustrating that the CBD is clear of calculi; D: MRCP showing no signs of CBD stones.

0.05). In contrast, in the anisodamine group, the rate of spontaneous passage of CBD stones in the first 2 wk using anisodamine was significantly higher than that in the latter 2 wk without anisodamine treatment ( $P < 0.05$ ), (Table 2). All 197 patients with symptomatic CBD stones experienced 1-3 attacks of biliary colic during follow-up. It was difficult to verify whether the stone passed during the colic attack or whether it passed silently.

Table 3 lists the results of univariate and multivariate

logistic regression models constructed to identify the predictors for spontaneous passage. Factors significantly increasing the possibility of spontaneous passage by univariate logistic regression analyses were the diameter of the CBD stone and anisodamine therapy. Furthermore, multivariate logistic regression analyses also revealed that the factors associated with spontaneous passage included the diameter of the CBD stone (OR = 3.095; 95%CI: 1.927-4.970;  $P = 0.000$ ) and anisodamine therapy (OR =

**Table 1 Baseline characteristics of patients *n* (%)**

	Control group ( <i>n</i> = 97)	Anisodamine group ( <i>n</i> = 100)	<i>P</i> value
Age (yr)	59.1 ± 10.9	58.0 ± 12.4	0.564
Sex			0.422
Male	42 (43.3)	49 (49.0)	
Female	55 (56.7)	51 (51.0)	
Main presentations			
Biliary colic	40 (41.2)	44 (44.0)	0.695
Cholangitis	25 (25.8)	27 (27.0)	0.845
Jaundice	20 (20.6)	19 (19.0)	0.776
Acute pancreatitis	12 (12.4)	10 (10.0)	0.597
Median time interval from symptom onset to hospital admission			0.606
< 24 h	84 (86.6)	89 (89.0)	
≥ 24 h and ≤ 48 h	13 (13.4)	11 (11.0)	
Imaging methods for diagnosis			0.918
Conventional CT	82 (84.5)	84 (84.0)	
MRCP	15 (15.5)	16 (16.0)	
Median diameter of CBD stone	0.54 ± 0.24	0.55 ± 0.24	0.837
Minimum, mm	0.3	0.3	
Maximum, mm	1.0	1.0	
Diameter of CBD stone			0.959
< 5 mm	44 (45.4)	45 (45.0)	
≥ 5 mm and ≤ 10 mm	53 (54.6)	55 (55.0)	
Median diameter of CBD	12.6 ± 4.3	12.9 ± 3.8	0.683
Minimum, mm	8.0	8.3	
Maximum, mm	23.0	25.0	
History of cholecystectomy			0.856
Yes	27 (27.8)	29 (29.0)	
No	70 (72.2)	71 (71.0)	

CT: Computed tomography; CBD: Common bile duct; MRCP: Magnetic resonance cholangiopancreatography.

3.534; 95%CI: 1.810-6.902; *P* = 0.000). Clinical presentation did not significantly influence the rate of spontaneous passage (Table 3).

Among the 69 patients whose CBD stones passed spontaneously, 50 patients with gallstones underwent LC and avoided CBD intervention, and the other 19 patients with a history of cholecystectomy did not receive additional treatment. Among the 117 patients who failed to pass CBD stones, 85 patients with gallstones underwent LC and laparoscopic CBD exploration (56 trans-ductal and 29 trans-CBD), and 32 patients with a history of cholecystectomy underwent ERCP/EST to remove CBD stones. No patient underwent conversion to open surgery. Exploration of the CBD and common hepatic duct in all of these patients found stones, which were removed using a Dormia basket or flushed into the duodenum. The CBD was closed over an appropriately sized T-tube in 29 patients by incising and opening the CBD directly with stone retrieval after the stones were removed under endoscopic visualization. T-tube cholangiography was undertaken 21 d or later postoperatively, and the T-tube was removed if abnormalities were not observed.

Tachyarrhythmia, palpitations, blurred vision and severe retention of urine were not observed in the anisodamine group. Of 100 patients in the anisodamine group, 11 patients experienced a dry mouth occasionally, which disappeared immediately after withdrawal of anisodamine.

**Table 2 Rate of spontaneous passage of common bile duct stones**

Diameter of CBD stone	Control group ( <i>n</i> = 97)	Anisodamine group ( <i>n</i> = 100)	<i>P</i> value (intergroup)
< 5 mm	31.8% (15/44)	71.1% (31/45)	0.001
≥ 5 mm and ≤ 10 mm	15.1% (7/53)	27.3% (16/55)	0.044
Total	22.7% (22/97)	47.0% (47/100)	0.000
Within-group <i>P</i>	0.014	0.000	
Week 1	5.2% (5/97)	17.0% (17/100)	0.008
Week 2	5.4% (5/92)	28.9% (24/83)	0.000
Week 3	6.9% (6/87)	5.1% (3/59) <sup>a,c</sup>	0.740
Week 4	7.4% (6/81)	5.4% (3/56) <sup>a,c</sup>	0.737
Total	22.7% (22/97)	47.0% (47/100)	0.000
Within-group <i>P</i>	0.907	0	

<sup>a</sup>*P* < 0.05 vs week 1; <sup>c</sup>*P* < 0.05 vs week 2. CBD: Common bile duct.

## DISCUSSION

This study was designed to investigate the rate of spontaneous passage of single and symptomatic CBD stones with the aid of a 2-wk course of anisodamine in symptomatic patients to demonstrate the role of conservative treatment for symptomatic CBD stones. The results of the study showed that 47.0% (47/100) of CBD stones passed spontaneously in the anisodamine group, and 87.2% (41/47) of the passages occurred in the first 2 wk with anisodamine treatment. However, only 22.7% (22/97) of patients passed CBD stones spontaneously in the control group, with comparable rates of spontaneous passage each week. Multivariate analyses indicated that anisodamine therapy carried a substantial advantage for accelerating the spontaneous passage of CBD stones. Moreover, stones < 5 mm in diameter were more likely to pass spontaneously, and spontaneous passage was not related to the clinical presentation. This finding would be useful for clinicians to predict which patients might pass stones spontaneously and to make rational clinical judgments.

Most clinicians specializing in hepatobiliary medicine are familiar with spontaneous passage of CBD stones, and such passage is recognized in the literature<sup>[1,13-16]</sup>. Collins *et al*<sup>[1]</sup> reported that 12/34 of silent CBD stones confirmed by intraoperative cholangiography in selective LC passed spontaneously 6 wk after the procedure. Frossard *et al*<sup>[13]</sup> evaluated the prevalence and time-course of CBD stone passage in symptomatic patients by analyzing discrepancies between endoscopic ultrasonography and ERCP as a function of the time elapsed between these two procedures. They found that the rate of spontaneous passage of CBD stones was 21% (12/57), and that the rates of spontaneous passage in different periods (from 6 h to 3 d and from 3 to 27 d) were 21% (8/37) and 20% (4/20), respectively. They also concluded that stone diameter was the only factor that predicted passage, and that the rate of spontaneous passage of stones with a diameter of > 8 mm was only 4.3% (2/47). Tranter *et al*<sup>[14]</sup> conducted a study in 1000 patients to determine the rate of spontaneous passage of CBD stones and related it

**Table 3** Factors associated with spontaneous passage of common bile duct stones in univariate and multivariate logistic regression models

Parameters	Univariate analyses			Multivariate analyses		
	OR	95%CI	P value	OR	95%CI	P value
Age	1.141	0.735-1.724	0.534			
Sex	0.980	0.545-1.763	0.947			
Main presentation	1.001	0.754-1.328	0.994			
Diagnosis by imaging method	0.976	0.438-2.177	0.954			
Diameter of CBD stone	2.847	1.819-4.458	0.000	3.095	1.927-4.970	0.000
Median diameter of CBD	1.331	0.726-2.041	0.342			
History of cholecystectomy	1.070	0.557-2.054	0.839			
Anisodamine treatment	3.023	1.632-5.600	0.000	3.534	1.810-6.902	0.000
Median time interval from onset to hospital admission	0.596	0.252-1.412	0.240			

CBD: Common bile duct.

to the various presentations of CBD stones. They found that 390/532 CBD stones passed spontaneously, but they did not specify the observation period. Lefemine *et al*<sup>[15]</sup> retrospectively investigated 108 patients presenting with jaundice due to CBD stones, and found that spontaneous passage of CBD stones occurred in 60 (55.6%) of 108 patients within approximately 4 wk. The inclusion criteria of the latter two studies<sup>[14,15]</sup> were not strict: patients with a history of jaundice, pancreatitis, abnormal results of liver function tests, or a dilated CBD were assumed to have a history of CBD stones. Therefore, the reported rates of spontaneous passage were probably overestimated.

Reported rates of spontaneous passage of CBD stones vary mainly because of different inclusion criteria and research methods used<sup>[1,13-15]</sup>. However, studies have demonstrated that a significant portion of CBD stones can pass spontaneously through the sphincter of Oddi into the duodenum with conservative treatment. However, further investigation is required to clarify the true potential of spontaneous passage of symptomatic CBD stones of different diameters because this potential is related closely to clinical decision-making for patients with symptomatic CBD stones. The present study was the first randomized study to investigate the rate of spontaneous passage in selected patients with CBD stones with the aid of a 2-wk course of anisodamine, and no studies have focused on other pharmacologic therapies for CBD stones. We showed not only the rate of spontaneous passage of CBD stones  $\leq 10$  mm in the control arm, but also confirmed that anisodamine effectively and safely accelerated the spontaneous passage of CBD stones in these patients.

Tranter *et al*<sup>[14]</sup> found that spontaneous passage of CBD stones occurred more commonly in patients with pancreatitis, biliary colic, and cholecystitis, but less commonly in jaundiced patients. They explained that patients with jaundice may be treated more quickly than other patients, allowing less time for spontaneous passage. The present study did not support this finding. We did not identify a particular characteristic of the patients who failed to pass stones spontaneously during the same observation period. Therefore, we concluded that spontane-

ous passage was not related to the presentation.

The limitation of this study was that the attending physicians were not blinded to the patient allocation. To address this, all physicians underwent strict and standardized training based on the treatment protocol. This training stressed that all patients should be treated with the same demeanor regardless of their randomized treatment arm.

In conclusion, 2 wk of anisodamine administration can safely accelerate spontaneous passage of single and symptomatic CBD stones  $\leq 10$  mm in diameter in symptomatic patients. These findings suggest that conservative treatment may be regarded as first-line management for these patients, especially for those with a stone  $< 5$  mm.

## COMMENTS

### Background

Common bile duct (CBD) stones are known to pass spontaneously in many patients. This phenomenon has not been given sufficient emphasis in terms of optimizing the timing of management of CBD stones. Anisodamine has been used as an antispasmodic agent for relieving colic pain in the treatment of CBD stones in China for more than 40 years. The authors hypothesized that anisodamine therapy can accelerate the passage of CBD stones by relaxing the sphincter of Oddi. The rate of spontaneous passage of single and symptomatic CBD stones  $\leq 10$  mm in 4 wk with or without a 2-wk course of anisodamine, was investigated.

### Research frontiers

The management of CBD stones has focused mainly on the choice of treatment rather than its timing. Consensus on the timing of management of symptomatic CBD stones is lacking. Most physicians, surgeons and endoscopists propose that symptomatic CBD stones should be extracted promptly. However, accumulating evidence has shown that CBD stones often pass spontaneously, which raises the possibility of avoiding CBD intervention.

### Innovations and breakthroughs

The results of the present study suggested that anisodamine administration for 2 wk can safely accelerate spontaneous passage of single and symptomatic CBD stones  $\leq 10$  mm in symptomatic patients. These findings indicated that conservative treatment could be the first-line management for these patients, especially for those with stones  $< 5$  mm in diameter.

### Applications

The results of the present study suggest that 2 wk of anisodamine administration can safely accelerate spontaneous passage of single and symptomatic CBD stones  $\leq 10$  mm in diameter in symptomatic patients. These findings indicated that conservative treatment could be the first-line management for these patients, especially for those with stones  $< 5$  mm.

**Peer review**

The authors undertook a randomized controlled trial investigating if anisodamine accelerated spontaneous passage of single symptomatic CBD stones  $\leq$  10 mm in diameter. The study was well conducted and the results are interesting that 47.0% of CBD stones  $\leq$  10 mm in diameter passed spontaneously with the aid of a 2-wk course of anisodamine.

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## Salvage irrigation-suction in gracilis muscle repair of complex rectovaginal and rectourethral fistulas

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### Abstract

**AIM:** To evaluate the efficacy of gracilis muscle transposition and postoperative salvage irrigation-suction in the treatment of complex rectovaginal fistulas (RVFs) and rectourethral fistulas (RUFs).

**METHODS:** Between May 2009 and March 2012, 11 female patients with complex RVFs and 8 male patients with RUFs were prospectively enrolled. Gracilis muscle transposition was undertaken in all patients and postoperative wound irrigation-suction was performed in patients with early leakage. Efficacy was assessed in terms of the success rate and surgical complications. SF-36 quality of life (QOL) scores and Wexner fecal incontinence scores were compared before and after surgery.

**RESULTS:** The fistulas healed in 14 patients after gracilis muscle transposition; the initial healing rate was 73.7%. Postoperative leakage occurred and continuous irrigation-suction of wounds was undertaken in 5 patients: 4 healed and 1 failed, and postoperative fecal diversions were performed for the patient whose treatment failed. At a median follow-up of 17 mo, the overall healing rate was 94.7%. Postoperative complications occurred in 4 cases. Significant improvement was observed in the quality outcomes framework scores ( $P < 0.001$ ) and Wexner fecal incontinence scores ( $P = 0.002$ ) after the successful healing of complex RVFs or RUFs. There was no significant difference in SF-36 QOL scores between the initial healing group and irrigation-suction-assisted healing group.

**CONCLUSION:** Gracilis muscle transposition and postoperative salvage wound irrigation-suction gained a high success rate in the treatment of complex RVFs and RUFs. QOL and fecal incontinence were significantly improved after the successful healing of RVFs and RUFs.

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**Key words:** Rectovaginal fistula; Rectourethral fistula; Gracilis muscle; Quality of life; Therapeutic irrigation

**Core tip:** A prospective study of 19 patients with complex rectovaginal fistulas (RVFs) and rectourethral fistulas (RUFs) undergoing gracilis muscle transposition followed by postoperative salvage wound irrigation-suction was reported to yield an overall healing rate of 94.7%. In addition, quality of life and fecal incontinence were reported to be significantly improved after the successful healing of RVFs and RUFs.

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## INTRODUCTION

Rectovaginal fistulas (RVFs) and rectourethral fistulas (RUFs) are pathological sinus tracts between the rectum and vagina or urethra. The exact incidence of RVFs/RUFs is not known. The reported frequency of RVFs following low anterior rectal resection reached 10%<sup>[1-5]</sup>, and RUFs developed in 0.53% of patients after radical prostatectomy<sup>[6]</sup>. Moreover, such fistulas have attracted appreciable attention because of their obvious influence on quality of life (QOL) and their difficulty of repair. Multiple surgical procedures to repair these fistulas have been described, but the success rates have varied dramatically, and the sample sizes reported were generally small<sup>[7]</sup>. The most popular treatments are transrectal or transvaginal flaps, direct peritoneal closure, a wide variety of abdominal procedures (including coloanal anastomosis and tissue interposition), and gracilis muscle transposition<sup>[8,9]</sup>.

Gracilis muscle transposition was first described in 1928 by Garlock<sup>[10]</sup>, who rotated the muscle subcutaneously to the fistula region. The treatment was subsequently applied in patients with vesicovaginal fistulas in 1952<sup>[11]</sup>. This method satisfies the criteria of relative safety afforded by perineal procedures while providing the healthy, well-vascularized tissue offered by abdominal procedures<sup>[12]</sup>.

After reviewing the results of various repairs (including gracilis muscle transposition for RVFs and RUFs)<sup>[13]</sup>, Wexner *et al*<sup>[11]</sup> reported that gracilis muscle transposition is associated with minimal morbidity and a high success rate, but a second repair is needed in some cases. The leakage of anastomoses in the digestive tract was found to be the predominant reason for postoperative fistulization<sup>[14]</sup>; anastomoses may be cured without surgical intervention by continued irrigation-suction<sup>[15]</sup>.

In the present study, we hypothesized the following: (1) gracilis muscle transposition to repair complex RVFs and RUFs can improve QOL; and (2) continuous wound irrigation-suction during the perioperative period can avoid the need for a second repair and can yield a high success rate.

## MATERIALS AND METHODS

### *Ethical approval of the study protocol*

The study protocol was approved by the Ethics Committee of Capital Medical University (Beijing, China). All the patients provided written informed consent before being enrolled in the study.

### *Patient characteristics and study design*

The prospectively registered data were as follows: patient demographics, etiology, clinical manifestation, diagnostic

procedures, repair history, use of fecal and/or urinary diversions, fistula type, surgical details, morbidity, and preoperative management. The Wexner score<sup>[16]</sup> and the Short-Form (SF)-36 score<sup>[17]</sup> were recorded preoperatively and 6 mo postoperatively by an independent research assistant.

Inclusion criteria and exclusion criteria were: All patients with RUFs were enrolled. However, complex RVFs had to meet one or more of the following criteria: (1) positioning in the upper-third of the rectovaginal septum; (2) size of fistula orifice  $\geq 2.5$  cm; (3) secondary to inflammatory bowel disease, radiotherapy or a tumor; and (4) having experienced a previous failed repair.

### *Surgical procedure*

The repair of complex RVFs and RUFs *via* gracilis muscle transposition was conducted as described previously<sup>[18]</sup>. At the end of surgery, two draining tubes were placed in perineal and leg wounds. All leakages were confirmed as fistula repair failures using the methylene blue test<sup>[19]</sup>. The perineal tube was routinely replaced with an irrigation-suction device (Changzhou Anker Medical Co., Ltd, Changzhou, China) as soon as leakage was observed from the vagina or from the perineal incision (Figure 1). The procedure was conducted by one surgeon, and local anesthesia was not used. Continuous irrigation with approximately 2000-3000 cm<sup>3</sup> of 0.9% saline per day and suction with a negative pressure of 0-0.01 kPa were used for all patients with leakages. The criterion for ending irrigation-suction was that suction collection was clear for  $> 3$  d.

Fecal diversions were used for 3 mo prior to gracilis interposition in 15 patients with RUFs and RVFs and were removed 3 mo after healing was confirmed. Epicystostomy was undertaken in 7 patients with RUFs before gracilis interposition and was closed 3 mo after healing was confirmed. A urinary catheter was left in place for 1 mo after the RUF procedures. The criteria for successful repair were that the fistula healed within 1 mo postoperatively or from the completion of irrigation-suction. Follow-up began 3 mo after the last hospitalization.

### *Statistical analysis*

Quantitative data were presented as the mean and standard deviation or the median and interquartile range (IQR) depending on whether an underlying normal distribution can be assumed. Numeric data were presented as percentages. Score values were compared using Student's *t* test or corresponding nonparametric methods. All the tests were two sided, and  $P < 0.05$  was considered to be of statistical significance.

## RESULTS

From May 2009 to November 2011, 18 patients (8 RUFs and 11 complex RVFs) were recruited into the study; 10 patients were excluded (6 because they had simple RVFs and 4 because they underwent procedures other than gracilis muscle transposition).

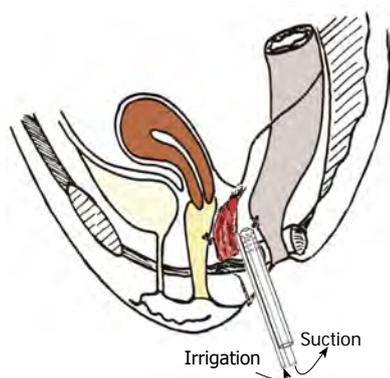


Figure 1 An irrigation-suction device was placed in the leakage site following gracilis muscle transposition.

The median age of the study cohort was 47 years (range, 16-80 years). The median value of the body mass index (BMI) was 21.9 kg/m<sup>2</sup> (range, 15.6-29.1 kg/m<sup>2</sup>). The median duration of the disease was 18 mo (range, 13-48 mo). The etiologies of the RUFs were previous surgery and pelvic irradiation of prostatic carcinoma ( $n = 5$ ), congenital imperforation ( $n = 1$ ), bulbourethral infection ( $n = 1$ ) and pelvic injury ( $n = 1$ ). The etiologies of the complex RVFs were surgery for rectal cancer ( $n = 4$ ), gynecologic surgery ( $n = 3$ ), birth trauma ( $n = 3$ ) and pelvic injury ( $n = 1$ ). The mean number of failed attempts was 1 (0-3). Fistulas associated with Crohn's disease were not observed in this cohort.

All fistulas were in the upper external sphincter and had a mean diameter of 1.6 cm (range, 0.5-3.0 cm). The median duration of surgery was 247 min (range, 120-400 min). The median postoperative hospital stay was 21 d (range, 10-39 d). The median postoperative follow-up was 17 mo (range, 6-34 mo). Postoperative short-term complications were numbness and pain in the thigh ( $n = 2$ ) and leg numbness ( $n = 2$ ), and both normalized within 6 mo without surgical intervention. No long-term complications were reported. The initial success rate of fistula healing after gracilis muscle transposition was 73.7% (14/19).

As an early sign of repair failure, postoperative leakage occurred in 5 patients (one male and 4 female). The median age was 53 years (range, 30-75 years). The median time from gracilis muscle transposition to the first leakage was 7 d (range, 6-10 d). Four patients were healed after continuous irrigation-suction, and the median duration of irrigation-suction was 8 d (range, 4-27 d). The overall success rate was 94.7% (18/19). The failed case was a 30-year-old female with RVF following 2 failed repairs. Gracilis muscle transposition was undertaken without preoperative fecal diversion; leakage occurred 6 d after surgery; irrigation-suction was conducted for 27 d and a sigmoid colon stoma was made 12 d postoperatively. However, the fistula persisted during a follow-up period of 22 mo.

The median Wexner score for successfully repaired fistulas was 15 (IQR = 18) and 0 (IQR = 0) before and

6 mo after the surgery, respectively. Fecal incontinence improved significantly after successful repair ( $P = 0.002$ ). QOL improved significantly after successful healing ( $P < 0.001$ ; Table 1). No difference was found in QOL in subjects whose fistula repair was successful ("initial success group") and those healing was successful after irrigation-suction ("secondary success group"; Table 2).

## DISCUSSION

The success rate of RUF/RVF repair after gracilis muscle transposition has been reported to be between 77% and 90%<sup>[18,20-23]</sup>. Wexner *et al*<sup>[11]</sup> reported the largest series of 53 RVFs and RUFs with gracilis muscle interposition, and the overall initial success rate was 70%; after seven repeated gracilis muscle transpositions, the final success rate was 87%. In the present study, the initial success rate was 73.7%; after continuous irrigation-suction, the success rate increased to 94.7%. Repeated gracilis muscle transposition was not performed in the present study. Continuous irrigation-suction was effective in improving the success rate of gracilis muscle transposition for complex RVFs and RUFs, and it protected patients from the potential complications associated with a second repair.

QOL is one of the most important outcomes of RVF/RUF repair. However, data related to changes in the QOL of patients with RVFs/RUFs and to the improvement of QOL after repair are lacking. This is the first prospective study to compare the preoperative and postoperative SF-36 scores in RVF/RUF patients after gracilis muscle transposition. Our data showed that the QOL improved significantly after successful RUF/RVF repair. The QOL was similar between the initial success group and secondary success group after irrigation-suction. Successful healing of RUFs/RVFs, after either surgical repair or continuous irrigation-suction, was the key factor in improvement of QOL. Correlations were found between the Wexner score and the SF36 domain scores in patients with pelvic disorders such as urinary incontinence and pelvic organ prolapse<sup>[24]</sup>. The fecal incontinence score increased significantly after successful healing of RVFs/RUFs in the current study, which may have contributed to the improvement in QOL. However, Lefèvre *et al*<sup>[25]</sup> reported that patients with recurrent RVFs have significantly lower postoperative SF-36 scores compared with the general population, while the impact of RVF/RUF repair on QOL remained unknown because the authors did not compare QOL before and after successful repair. Samplaski *et al*<sup>[26]</sup> investigated the QOL outcomes in patients undergoing transperineal repair with gracilis muscle interposition for RUF and achieved reasonable bowel and bladder function postoperatively. Further studies aimed at QOL in patients with RVFs or RUFs, especially whether QOL is improved even when fecal incontinence remains unchanged after repair, are needed to provide consensus for clinical care.

An adequate length of the bulky muscular portion of a well-vascularized gracilis to place the muscle between

**Table 1 Comparison of SF-36 scores before and after gracilis muscle transposition for complex rectovaginal fistulas and rectourethral fistulas**

Items	Preoperation (n = 18)		Postoperation (n = 18)		P value
	Median	IQR	Median	IQR	
Physical functioning	65	25	95	5	< 0.001
Role limitations-physical	13	25	100	0	< 0.001
Body pain	52	36	90	9	< 0.001
General health	34	19	87	16	< 0.001
Vitality	50	16	90	6	< 0.001
Social functioning	44	25	100	16	< 0.001
Role limitation-emotional	17	33	100	0	0.001
Mental health	44	32	92	6	< 0.001

IQR: Interquartile range. 0: Poorest; 100: Best.

**Table 2 SF-36 scores in the initial success group and the secondary success group after irrigation-suction**

Items	Initial success group (n = 14)		Secondary success group (n = 4)		P value
	Median	IQR	Median	IQR	
Physical functioning	95	8	95	4	0.277
Role limitation-physical	100	0	100	0	0.721
Body pain	90	16	90	5	0.645
General health	85	9	90	19	0.158
Vitality	90	15	88	13	0.798
Social functioning	100	28	100	9	0.505
Role limitation-emotional	100	0	100	0	0.721
Mental health	92	12	92	6	0.277

IQR: Interquartile range. 0: Poorest; 100: Best.

the two suture lines is important for initial success. It is important to place the tip of the irrigation-suction tube close to the leakage site for successful healing. Hence, the length of the irrigation-suction tube underneath the skin should be exactly the same as that of the original tube.

The success rate of gracilis muscle transposition for RUFs is, in general, higher than that for RVFs, and the success rate is considerably higher in non-Crohn’s disease-associated RVFs than in Crohn’s disease-associated RVFs<sup>[13]</sup>. Crohn’s disease may have a negative impact on fistula healing. In the present study, Crohn’s disease-associated RVF was not encountered, which might explain why that initial success rate and final success rate were considerably higher compared with previous studies. The efficacy of gracilis muscle transposition for Crohn’s-associated RVF needs further verification.

The main limitation of the present study was the low prevalence of postoperative leakage. The number of patients in the secondary success group (only 4) seems too small to provide a meaningful comparison to the initially success group; therefore, we should accept the lack of difference in QOL between the two groups with caution. Further studies comparing postoperative leakage treated with or without perioperative irrigation-suction are necessary before this surgical method can be widely used.

According to the data, gracilis muscle transposition may be indicated in complex RVFs/RUFs resulting from pelvic irradiation, surgical injury, trauma and infection, especially they are associated with malignancy and failed

repair. Postoperative irrigation-suction should be considered as soon as a leakage is found. Gracilis muscle transposition and postoperative salvage wound irrigation-suction to treat complex RVFs and RUFs was shown to have a high success rate. QOL and fecal incontinence were improved significantly after the successful healing of RVFs and RUFs.

**COMMENTS**

**Background**

The obvious influences of complex rectovaginal fistulas (RVFs) and rectourethral fistulas (RUFs) on quality of life (QOL) and the difficulty of their repairs have attracted extensive attention. Gracilis muscle repair is associated with minimal morbidity and a high success rate, but second repair is often required in some cases.

**Research frontiers**

The success rate of RUF/RVF repair after gracilis muscle transposition has been reported to be 77%-90%. Data related to changes in the QOL of patients with RVFs/RUFs and to the improvement of QOL after repair have not been reported. This is the first prospective study comparing the preoperative and postoperative SF-36 scores in RVF/RUF patients after gracilis muscle transposition. The data showed that the QOL improved significantly after successful repair of RUF/RVF.

**Innovations and breakthroughs**

In this study, the initial success rate was 73.7% and, after continuous irrigation-suction, the success rate increased to 94.7%. Repeated gracilis muscle transposition was not carried out in the study. In addition, QOL and fecal incontinence were first reported to significantly improve after successful healing of RVFs and RUFs.

**Applications**

Postoperative irrigation-suction should be considered as soon as leakage was

found.

### Terminology

QOL referred to the general well-being of individuals and societies, which is used in a wide range of contexts, including the fields of international development, healthcare, and politics. Within the field of healthcares, QOL is often regarded in terms of how it is negatively affected, on an individual level, a debilitating weakness that is not life-threatening, life-threatening illness that is not terminal, terminal illness, the predictable, natural decline in the health of an elder, an unforeseen mental/physical decline of a beloved one, chronic, end-stage disease processes, *etc.* RVFs referred to pathological sinus tracts between the rectum and vagina. RUFs referred to pathological sinus tracts between the rectum and urethra.

### Peer review

It is an innovative paper dealing with a relevant surgical intervention in fecal incontinence. Though this is a small series it does provide useful information to the readers.

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## Role of ABCG2 expression driven by cisplatin in platinum-containing chemotherapy for gastric cancer

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were assessed after cancer cells were incubated with cisplatin, and were divided into terciles and compared in relation to clinical outcomes.

**RESULTS:** Among groups classified by expression time of ABCG2 mRNA, no significant differences in baseline clinical characteristics and pathological findings were detected. The median overall time was 14.2 (95%CI: 9.7-18.6), 11.4 (95%CI: 6.3-16.5) and 8.1 (95%CI: 5.4-10.8) in patients with low, intermediate and high increases in ABCG2 mRNA expression times ( $P < 0.05$ ), respectively. Median survival associated with performance status and tumor node metastasis (TNM) stage showed a similar trend, with longer survival and higher risk for mortality associated with lower performance status score and TNM stage. In a multivariate analysis for survival with Cox proportional-hazards model, increased ABCG2 mRNA expression time was an independent predictor for overall survival. Overall survival was longer with increased ABCG2 mRNA expression times  $\leq 0.71$  than increased ABCG2 mRNA expression times  $> 0.71$ , with a hazard ratio for death of 0.855 (95%CI: 0.615-0.962,  $P = 0.038$ ).

**CONCLUSION:** Increased ABCG2 mRNA expression time driven by cisplatin is associated with survival of gastric cancer patients, and this may help modify the therapeutic strategies.

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**Key words:** Gastric cancer; ABCG2 mRNA expression; Cisplatin; Overall survival

**Core tip:** As a prognostic marker of poor clinical outcome, the high expression of ABCG2 has become a hot research topic and focus. Nevertheless, there are few studies involving the relevance of ABCG2 expression driven by chemotherapeutic agents and its clinical significance. This is the first study examining the impact of increased ABCG2 mRNA expression time driven by

### Abstract

**AIM:** To investigate the relationship between increases in expression time of ABCG2 mRNA driven by cisplatin and efficacy of platinum-containing chemotherapy for gastric cancer.

**METHODS:** Tumor specimens and normal control tissues were collected from 78 patients with gastric cancer treated from January 2008 to December 2011. Fresh tumor tissue obtained from the surgically resected specimens was tested within 6 h. Polymerase chain reaction products were run on 2% agarose gels and analyzed under ultraviolet light after ethidium bromide staining. Increases in ABCG2 mRNA expression time

cisplatin *in vitro* in gastric cancer and its relationship with overall survival of the patients.

Zhang Q, Li K, Xu JH, Zhao CG, Gao Q, Wu B, Liu XY. Role of ABCG2 expression driven by cisplatin in platinum-containing chemotherapy for gastric cancer. *World J Gastroenterol* 2013; 19(39): 6630-6636 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6630.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6630>

## INTRODUCTION

Gastric cancer is the fourth most prevalent malignant cancer worldwide, and is the second most frequent cause of cancer death<sup>[1]</sup>. Despite advances made in gastric cancer therapy, the prognosis of patients with gastric cancer remains unsatisfactory. Because of early detection in screening programs in Japan, survival is prolonged (52%), whereas survival in the United States, Europe, and China is only 20%-25% due to delayed diagnosis<sup>[2]</sup>. The 5-year survival rate for advanced or metastatic gastric cancer is nearly 5%-20%, with a median overall survival being less than 1 year<sup>[3,4]</sup>. With the development of new anticancer drugs, such as taxanes, CPT-11, oxaliplatin, gefitinib and S-1, significant improvements in the efficacy of chemotherapy against gastric cancer have been achieved<sup>[5]</sup>. However, some patients still fail on first-line chemotherapy and will relapse and eventually develop resistance to currently available treatment options due to the acquisition of multidrug resistance (MDR)<sup>[6]</sup>. Therefore, it is necessary to find markers which could accurately predict the risk of gastric cancer, and give the evidence for early prediction of the clinical outcome so as to improve the clinical management of gastric cancer patients.

Breast cancer resistance protein (BCRP/ABCG2), the second member of the ATP-binding cassette-transporter superfamily, is prominently expressed in the epithelium of small intestine and colon, liver canalicular membranes, ducts and lobules of mammary tissue and blood-brain barrier, which plays a pivotal role in the bioavailability and brain disposition of drugs<sup>[7]</sup>. A broad spectrum of anticancer drugs, sulfate and glucuronide conjugates of sterols and xenobiotics, natural compounds and toxins, fluorescent dyes, photosensitizers, and antibiotics have been identified as substrates of ABCG2<sup>[8]</sup>. As a major drug transporter, ABCG2 has been shown to play an active role in MDR in various cancers<sup>[9-11]</sup>. Increased expression of ABCG2 results in resistance to anticancer drugs, including topoisomerase inhibitors, anthracyclines, camptothecin (CPT) analogs, tyrosine kinase inhibitors (TKI), and antimetabolites<sup>[8]</sup>. The resistant phenotype is conferred through the reduction of cytoplasmic chemotherapeutic drug concentrations to levels below those required for cytotoxicity<sup>[7]</sup>. Using an immunohistochemical method, ABCG2 expression was seen in all 150 tumor samples comprising 21 types of cancer, especially

in carcinomas of the digestive tract (colon, esophagus and stomach), endometrium and lung, and in melanoma, which suggested that ABCG2 represented a common mechanism of clinical drug resistance<sup>[12]</sup>. Results from clinical studies indicate that high expression of ABCG2 in tumors is a prognostic marker of poor clinical outcome<sup>[9,13,14]</sup>. However, there are a limited number of studies on the relevance of ABCG2 expression driven by chemotherapeutic agents and its clinical significance.

In light of the above information, this study was an attempt to clarify the impact of increases in ABCG2 mRNA expression times driven by cisplatin on clinical outcome in patients with gastric cancer.

## MATERIALS AND METHODS

### Patients and samples

Tumor specimens and normal control tissues were collected from 78 patients with newly-diagnosed histologically proven gastric cancer (GC) in Shanghai Sixth People's Hospital, Renji Hospital and Shanghai Putuo Hospital, from January 2008 to December 2011. All of the tumor specimens were obtained before chemotherapy. None of the patients received preoperative radiotherapy or chemotherapy. The clinical stage of GC was determined on the basis of the tumor-node-metastasis (TNM) classification system recommended by the International Union Against Cancer. Demographic and clinicopathological details of the patients were collected from electronic patient records. The study was approved by the Institutional Review Board of Shanghai Sixth People's Hospital, Renji Hospital and Shanghai Putuo Hospital. All samples were obtained after receiving patients' written informed consent.

All patients received platinum-based first-line chemotherapy and second-line chemotherapy for GC according to the NCCN guideline of GC<sup>[6]</sup>. The platinum-based first-line were cisplatin 60-100 mg/m<sup>2</sup> plus fluoropyrimidine (750-1000 mg/m<sup>2</sup> *iv* on days 1-5 as a protracted continuous infusion, FP; *n* = 18), docetaxel (75 mg/m<sup>2</sup> *iv* on day 1, DP; *n* = 12), paclitaxel (135 mg/m<sup>2</sup> *iv* on day 1, PP; *n* = 10), capecitabine (1000 mg/m<sup>2</sup> *bid po* on days 1-14, XP; *n* = 25) or S-1 (40 mg/m<sup>2</sup> *bid po* on days 1-21, SP; *n* = 13). Chemotherapy was repeated every 3 wk (XP, DP, PP and FP) or every 5 wk (SP) according to the regimen. All the patients received 2 or more courses of chemotherapy or until the appearance of progressive disease. In second-line chemotherapy, patients were administered the following regimens: oral S-1 or capecitabine (*n* = 30); weekly paclitaxel (*n* = 18); irinotecan and cisplatin (*n* = 13); irinotecan and docetaxel (*n* = 9) and mitomycin C, etoposide and cisplatin (*n* = 8).

### Cell culture with cisplatin

Fresh tumor tissues obtained from the surgically resected specimens were tested within 6 h. The tumor tissue was cut into pieces (smaller than 1 mm<sup>3</sup>) and passed through No. 100 and No. 200 stainless steel meshes respectively

into a complete medium containing RPMI 1640 solution, 100 µg/mL penicillin, and 100 µg/mL streptomycin, and washed twice gently with the same solution. The viable cells were assessed using a trypan blue exclusion method. The cell suspension was collected into sterile 96-well flat-bottomed microtiter plates ( $1 \times 10^5$  cells per well) with or without 0.5 µg/mL cisplatin (CDDP). The plates were then incubated at 37 °C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub> for 72 h. Microtiter wells containing tumor cells but no anticancer agents were used as control cells, in which the total number of tumor cells was equivalent to that in the test wells.

### Quantitative polymerase chain reaction

Treated and untreated cancer cells were suspended at  $1 \times 10^6$  cells/mL in 5 mL PBS as samples. Total RNA was then isolated using TriPure Isolation reagent (Roche Diagnostics GmbH, Germany) or RNeasy Mini Kit (Qiagen, United States) according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) were performed using specific hydrolysis probes targeting ABCG2 on Applied Biosystems 7300S Real Time PCR systems (ABI, United States). Total RNA was isolated from cisplatin-treated GC cells using TRIzol (Tarkara, Dalian, China) and reverse-transcription was performed to synthesize cDNA using PrimeScript Reverse Transcriptase (Tarkara, Dalian, China). Subsequent PCR amplification was performed using 2 µg of cDNA under the following conditions: 95 °C for 30 s, 35 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (Tarkara, Dalian, China) was used. β-actin was used as an internal control. PCR products were run on 2% agarose gels and analyzed under ultraviolet light after ethidium bromide staining. The primer sequences of ABCG2 used in real-time RT-PCR were: sense 5'-TTTCCAAGC-GTTCATTC AAAAA-3', antisense 5'-TACGACTGT-GACA ATGATCTGAGC-3'. β-actin: sense 5'-ACC-GTG GAGAAGAGCTACGA-3', antisense 5'-GTACTT GCGCTCAGAAGGAG-3'. Each RT-PCR amplification was repeated in triplicate. The β-actin primer was included in every plate to avoid sample variations. The PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to check the PCR specificity. Each cDNA sample was triplicated and the corresponding no-RT mRNA sample was included as a negative control. The β-actin primer was included in every plate to avoid sample variations. The mRNA level of each sample was normalized to that of the β-actin mRNA. Relative increases in mRNA expression times of target gene against β-actin were measured as follows:  $\text{mRNA}_{\text{target}} = (\text{mRNA}_{\text{target}} / \text{mRNA}_{\beta\text{-actin}})_{\text{cisplatin treated cells}} / (\text{mRNA}_{\text{target}} / \text{mRNA}_{\beta\text{-actin}})_{\text{control cells}}$ . All data shown were the mean ± SD of three separate experiments.

### Statistical analysis

To determine the correlation between mRNA increased expression times and survival rate after chemotherapy, the  $\chi^2$  test or Fisher's exact test was used to analyze the

data with R software statistical environment (version 2.15.1; R Development Core Team, Vienna, Austria). Cumulative overall survival probability was calculated by the Kaplan-Meier method for censored failure time data, and statistical significance was calculated using the log-rank test for comparison of survival rates between the different groups. The Cox proportional-hazards model was used to calculate the hazard ratios.  $P < 0.05$  was considered statistically significant. All  $P$  values were two-tailed and unadjusted for potential multiple comparisons.

## RESULTS

### Patient characteristics

The clinical and pathological characteristics of the patients are outlined in Table 1. The median age was 59 years (range, 27-81 years). All of the patients were treated with platinum-based combination chemotherapeutic regimens. The median number of chemotherapy courses was 4 (range, 2-15). The disease progression was the most common reason for the discontinuation of the chemotherapy. The median follow-up time of the 78 patients was 12 mo (range, 3-42 mo).

ABCG2 mRNA levels were measured by quantitative PCR and increased expression times in the cancer cells incubated with cisplatin were calculated by comparing with cancer cells incubated without cisplatin. Using the method reported by Font *et al.*<sup>[15]</sup>, results from these analyses were used to categorize the patients into terciles according to their ABCG2 mRNA expression times (lowest tercile,  $\leq 0.71$ ,  $n = 40$ ; intermediate tercile, 0.71-1.80,  $n = 23$ ; and highest tercile,  $\geq 1.80$ ,  $n = 15$ ). No differences in clinical characteristics were observed according to BRCA1 mRNA expression levels. Among groups determined according to ABCG2 mRNA expression times, no significant differences in baseline clinical characteristics and pathological findings were detected (Table 1).

### ABCG2 mRNA increased expression times and clinical outcome

The median overall survival time was 14.2 mo (95%CI: 9.7-18.6), 11.4 mo (95%CI: 6.3-16.5) and 8.1 mo (95%CI: 5.4-10.8) in the patients with low, intermediate and high ABCG2 mRNA expression times (Table 2), respectively. Because survival rates were similar in patients with intermediate and high expression times, the patients in these two groups were mixed for further statistical analyses. In patients with intermediate/high ABCG2 mRNA expression times, median overall survival (OS) time was 10 mo (95%CI: 5.8-14.2), whereas in patients with low expression levels, median survival was 14.2 mo (95%CI: 9.7-18.6) ( $P = 0.025$ ) (Figure 1A). There was no statistical difference in progression-free survival (PFS) between the two groups (Figure 1B).

Univariate analysis was conducted to examine the impact of increases in ABCG2 mRNA expression times and other clinical pathological parameters on prognosis. In the univariate analysis, three parameters were found

**Table 1** Characteristics of patients according to increased expression times of ABCG2 mRNA by tertiles *n* (%)

Clinical pathological parameters	Total cohort	ABCG2 mRNA expression times			<i>P</i> value
		≤ 0.71 ( <i>n</i> = 40)	0.71-1.8 ( <i>n</i> = 23)	≥ 1.8 ( <i>n</i> = 15)	
Age (yr)					0.123
Median	59	58	61	59	
Range	27-81	27-79	28-81	30-80	
Gender					0.269
Female	19 (24.4)	10 (24.8)	6 (28.0)	3 (19.0)	
Male	59 (75.6)	30 (75.2)	17 (72.0)	12 (81.0)	
Performance status					0.556
0-1	70 (89.7)	36 (88.8)	20 (85.0)	14 (91.0)	
2	8 (10.3)	4 (11.2)	3 (15.0)	1 (9.0)	
Histological grad					0.106
Differentiated	39 (50.0)	19 (48.0)	12 (51.0)	8 (52.3)	
Undifferentiated	39 (50.0)	21 (52.0)	11 (49.0)	7 (47.7)	
Stage					0.170
III A	24 (30.8)	12 (29.0)	7 (31.0)	5 (33.0)	
III B	24 (30.8)	13 (32.1)	7 (30.0)	4 (28.0)	
IV	30 (38.5)	15 (38.9)	9 (39.0)	6 (39.0)	
Chemotherapeutic regimen					0.322
Cisplatin + fluoropyrimidine	18 (23.1)	9 (23.1)	6 (26.9)	3 (21.4)	
Cisplatin + docetaxel	11 (14.1)	6 (15.4)	3 (14.6)	2 (13.8)	
Cisplatin + paclitaxel	10 (12.8)	5 (12.8)	3 (15.1)	2 (14.3)	
Cisplatin + capecitabine	19 (24.4)	7 (32.1)	7 (30.5)	5 (35.8)	
Cisplatin + S-1	20 (25.6)	13 (16.7)	4 (12.8)	3 (14.7)	

**Table 2** Univariate analysis of median overall survival and hazard ratios for risk of mortality according to clinical parameters

Clinical pathological parameters	Overall survival		Risk of mortality	
	Month (95%CI)	<i>P</i> value	HR (95%CI)	<i>P</i> value
Performance status		0.012		
0-1	14.4 (10.8-17.0)		1.00 (referent)	
2	7.6 (2.5-13.7)		3.26 (0.96-16.46)	0.041
Histological grade		0.062		
Differentiated	15.5 (8.2-22.5)		1.00 (referent)	
Undifferentiated	10.9 (4.5-17.3)		4.95 (0.92-18.6)	0.122
Tumor-node-metastasis stage		0.001		
III A	19.3 (16.9-21.7)		0.87 (0.51-9.76)	< 0.001
III B	13.0 (7.8-18.2)		0.92 (0.81-2.15)	0.102
IV	7.2 (4.4-10.0)		1.00 (referent)	
Chemotherapeutic regimen		0.536		
Cisplatin + fluoropyrimidine	10.7 (5.1-16.3)		1.00 (referent)	
Cisplatin + docetaxel	13.2 (7.0-19.4)		0.84 (0.76-1.79)	0.073
Cisplatin + paclitaxel	12.6 (6.4-18.9)		0.91 (0.85-2.41)	0.145
Cisplatin + capecitabine	11.3 (5.6-18.0)		0.98 (0.90-3.26)	0.210
Cisplatin + S-1	12.0 (6.1-17.9)		0.95 (0.89-2.84)	0.179
ABCG2 mRNA expression times		0.031		
≤ 0.71	14.2 (9.7-18.6)		0.71 (0.43-0.96)	< 0.001
0.71-1.8	11.4 (6.3-16.5)		0.94 (0.76-1.52)	0.083
≥ 1.8	9.0 (6.4-11.6)		1.00 (referent)	

to be significantly associated with overall survival and risk for mortality statistically: performance status; TNM stage; and ABCG2 mRNA expression times (Table 2). Median survival associated with performance status and TNM stage showed a similar trend, with longer survival and higher risk for mortality associated with lower performance status score and TNM stage. In the multivariate analysis using the Cox proportional hazards model, only increased ABCG2 mRNA expression times and TNM stage were independent predictors for overall survival. The HR of TNM stage III A *vs* III B-IV for overall survival was 0.921 (95%CI: 0.656-0.983, *P* = 0.045), and

the HR of ABCG2 mRNA increased expression times ≤ 0.71 *vs* > 0.71 was 0.855 (95%CI: 0.615-0.962, *P* = 0.038).

## DISCUSSION

Platinum-containing regimens have now been clinically validated as effective advanced GC treatments. However, the relatively rapid acquired chemotherapeutic resistance to such therapies significantly limits their effects and remains a substantial obstacle to the clinical management of GCs. With the elucidation of molecular mechanisms of resistance, new strategies of predicting the formation

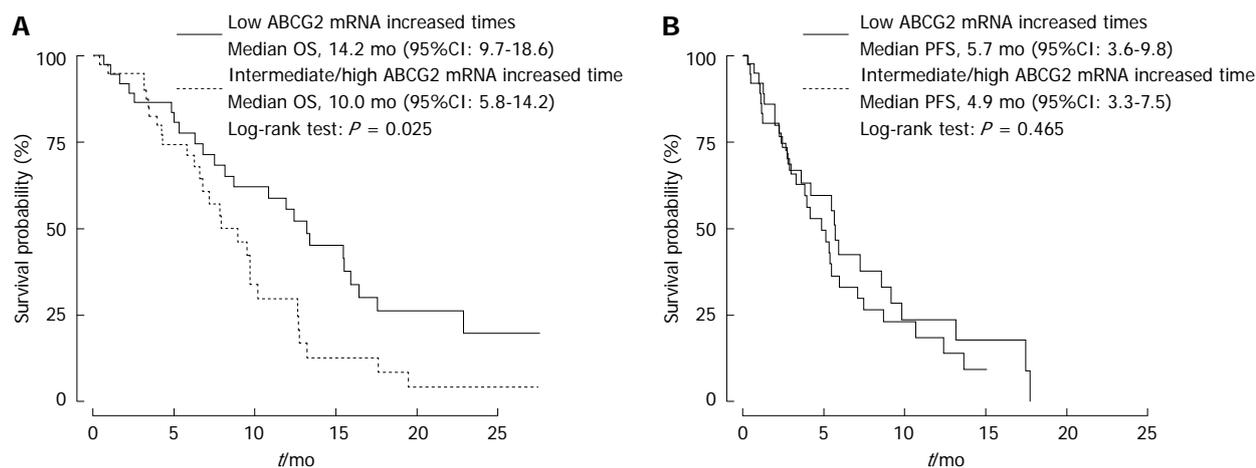


Figure 1 Overall survival (A) and progression-free survival (B) according to increased ABCG2 mRNA expression times (intermediate or high vs low tertiles). OS: Overall survival; PFS: Progression-free survival.

of resistance have become a potential area of interest. In the current study, we found that ABCG2 mRNA expression levels were increased by incubation in sub-therapeutic concentrations of cisplatin *in vitro*, which could lead to the MDR phenotype associated with ABCG2. Moreover, a significant relationship between increased ABCG2 mRNA expression times and overall survival was observed in GC patients treated with platinum-containing chemotherapy. Patients with higher ABCG2 mRNA expression times had a shorter median overall survival time. In a multivariate analysis, increased ABCG2 mRNA expression times driven by cisplatin *in vitro* might be an independent prognostic marker of overall survival, while no baseline clinical pathological parameters, except TNM stage, had a prognostic impact on overall survival. To the best of our knowledge, there has been no study examining the role of ABCG2 mRNA expression times induced by cisplatin *in vitro* in GC treatment.

ABCG2 has been found to be an important molecule involved in both innate and acquired MDR by regulating drug bioavailability. Over-expression of ABCG2 shows the potential to be an independent prognostic marker of both hematopoietic and solid malignancies. In hematopoietic malignancies, the results from several follow-up studies have validated the relationship between ABCG2 expression and the prognosis and survival of acute myeloid leukemia patients<sup>[16-18]</sup>. In esophageal squamous cell carcinoma, lung cancer, digestive tract tumors and lymphoma, the presence of ABCG2-positive cells in the tumor indicated by immunohistochemical study was associated with poorer survival<sup>[12,19-21]</sup>. These studies observed the relationship between the prognosis and the presence of chemotherapeutic resistance in tumors. However, they did not take the potential of acquisition of MDR into account. In fact, sub-therapeutic concentrations of anti-cancer agents, including cisplatin, would result in MDR in neoplastic cells<sup>[22]</sup>. The current study might provide a helpful method to assess the potential of acquisition of MDR driven by chemotherapeutic agents.

The expression of ABCG2 in normal and cancer cells appears to be regulated at different levels including gene amplification, epigenetic modifications, transcriptional and post-transcriptional regulation<sup>[8,23]</sup>. Nuclear hormone receptor proteins, including the pregnane X receptor (PXR), constitutive androstane receptor, and farnesoid X receptor have shown the ability to regulate the expression of ABC transporters<sup>[24]</sup>. In peripheral blood mononuclear cells and small intestine, PXR-selective ligands have been shown to increase the expression of various transporters like solute carrier family 21A6 (SLC21A6), ABCC2 and ABCB and significant relationships between expression of PXR and ABCB1, ABCC2 and ABCG2 have been reported<sup>[25-27]</sup>. One recent study found that IL-1 $\beta$  and TNF- $\alpha$  induced ABCG2 and PXR expression and NF- $\kappa$ B activity in some breast cancer and normal cell lines, which indicated a probable relationship between ABCG2, PXR and NF- $\kappa$ B<sup>[25]</sup>. Pradhan *et al*<sup>[28]</sup> showed that the cooperative binding of ER and p65 at adjacent response elements led to a major increase in both ABCG2 mRNA and protein expressions. Wu *et al*<sup>[29]</sup> reported that prolactin could up-regulate ABCG2 in T-47D human breast cancer epithelial cells *via* activation of JAK2/STAT5, MAPK, and PI3K signaling. However, the precise mechanism of regulating the expression of ABCG2 caused by DNA damage remains unclear.

In conclusion, ABCG2 mRNA increased expression times driven by cisplatin *in vitro* is associated with clinical outcome, which may be a novel biological marker for optimizing the treatment of GC. However, our data should be interpreted cautiously because of the limited number of patients enrolled. This is the first report to indicate a relationship between the potential for acquisition of MDR and prognosis of patients with GC. Further prospective randomized controlled trials with a larger number of patients would be worth doing to confirm these results, and the mechanism of DNA damage caused by cisplatin also should be elucidated.

## COMMENTS

**Background**

Gastric cancer (GC) ranks the fourth in morbidity among malignant tumors worldwide. Despite the advances made in diagnosis and treatment, the prognosis of GC patients remains poor. This is most probably attributed to the delayed diagnosis and resistance to currently available agents. Therefore, determining the markers which could predict the evidence for early clinical outcome and modify the therapeutic regimens is important.

**Research frontiers**

ABCG2 has been shown to represent a common mechanism of multidrug resistance in various cancers. Increasingly, it has become a hot research topic to identify the relevance of ABCG2 expression driven by chemotherapeutic agents and its clinical significance.

**Innovations and breakthroughs**

To date, there have been a limited number of studies regarding the potential for acquisition of MDR. In this study, the authors employed a method to assess the potential for acquisition of MDR driven by chemotherapeutic agents. Furthermore, the authors confirmed the significant correlation between the over-expression of ABCG2 and decreased overall survival rate.

**Applications**

By identifying the high expression of ABCG2 driven by cisplatin *in vitro* as being associated with overall survival, the authors evaluated the biological features and prognosis in GC, which could improve their understanding of GC, and provide a novel biological marker for optimizing the treatment of GC.

**Terminology**

ATP-binding cassette sub-family G member 2 is a protein that in humans is encoded by the ABCG2 gene. This protein transports various molecules across extra- and intra-cellular membranes, which may also play a role in multi-drug resistance to chemotherapeutic agents.

**Peer review**

The authors determined the expression of ABCG2 driven by cisplatin *in vitro*, and identified the high expression of this protein was associated with the prognosis of patients with GC. The result is interesting and indicates that the over-expression of ABCG2 could be used as a biomarker for prognosis in GC.

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## Expression and significance of Musashi-1 in gastric cancer and precancerous lesions

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### Abstract

**AIM:** To investigate expression of stem cell marker Musashi-1 (Msi-1) in relationship to tumorigenesis and progression of intestinal-type gastric cancer (GC).

**METHODS:** Endoscopic biopsy specimens and surgical specimens were obtained, including 54 cases of intestinal-type GC, 41 high-grade intraepithelial neoplasia, 57 low-grade intraepithelial neoplasia, 31 intestinal metaplasia, and 36 normal gastric mucosa. Specimens were fixed in 10% paraformaldehyde, conventionally dehydrated, embedded in paraffin, and sliced in 4- $\mu$ m-thick

serial sections. Two-step immunohistochemical staining was used to detect Msi-1 and proliferating cell nuclear antigen (PCNA) expression. Correlation analysis was conducted between Msi-1 and PCNA expression. The relationship between Msi-1 expression and clinicopathological parameters of GC was analyzed statistically.

**RESULTS:** There were significant differences in Msi-1 and PCNA expression in different pathological tissues ( $\chi^2 = 15.37, P < 0.01$ ;  $\chi^2 = 115.36, P < 0.01$ ). Msi-1 and PCNA-positive cells were restricted to the isthmus of normal gastric glands. Expression levels of Msi-1 and PCNA in intestinal metaplasia were significantly higher than in normal mucosa ( $U = 392.0, P < 0.05$ ;  $U = 40.50, P < 0.01$ ), whereas there was no significant difference compared to low or high-grade intraepithelial neoplasia. Msi-1 and PCNA expression in intestinal-type GC was higher than in high-grade intraepithelial neoplasia ( $U = 798.0, P < 0.05$ ;  $U = 688.0, P < 0.01$ ). There was a significantly positive correlation between Msi-1 and PCNA expression ( $r_s = 0.20, P < 0.01$ ). Msi-1 expression in GC tissues was correlated with their lymph node metastasis and tumor node metastasis stage ( $\chi^2 = 12.62, P < 0.01$ ;  $\chi^2 = 11.24, P < 0.05$ ), but not with depth of invasion and the presence of distant metastasis.

**CONCLUSION:** Msi-1-positive cells may play a key role in the early events of gastric carcinogenesis and may be involved in invasion and metastasis of GC.

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**Key words:** Musashi-1; Stem cells; Gastric cancer; Precancerous lesions; Immunohistochemistry.

**Core tip:** Gastric cancer (GC) is currently thought to be a disease originating in stem cells. We detected expression of stem cell marker Musashi-1 (Msi-1) and proliferating cell nuclear antigen (PCNA) in intestinal-type GC and precancerous lesions. Expression of Msi-1 and

PCNA in precancerous lesions was significantly higher than in normal mucosa, but lower than in intestinal-type GC. Msi-1 expression in GC tissues was correlated with lymph node metastasis and tumor node metastasis stage. These results suggest that expansion of Msi-1-positive cells is an early event in gastric carcinogenesis and may be involved in invasion and metastasis of GC.

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## INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death worldwide. Intestinal-type GC is believed to arise from an intestinal metaplasia to intraepithelial neoplasia sequence in gastric epithelial cells. The cancer stem cell theory indicates that cancers contain tumor-initiating stem cells possess the capacity for self-renewal and can cause the heterogeneous lineages of cancer cells constituting the tumor<sup>[1]</sup>. There is consensus that all gastric mucosal cells originate from stem cells<sup>[2]</sup>. The classical multistep model of gastric carcinogenesis requires a cell to receive multiple "hits" before it is transformed. Stem cells are long-lived; therefore, they are more likely to acquire these multiple hits and become transformed<sup>[3]</sup>. It was proposed that tumor-initiating cancer stem cells may be derived from normal stem cells; however, the lack of useful markers has made it difficult to characterize the stem cells in the human stomach and has hindered the study of the origin of GC.

Musashi-1 (Msi-1), an RNA-binding protein isolated as a mammalian homolog of a *Drosophila* protein, is selectively expressed in murine and human neural progenitor cells, including neural stem cells, can be used as a neural stem/progenitor cell marker, and plays an important role in the asymmetric division of neural stem cells<sup>[4]</sup>. In recent years, Msi-1 was also found to be expressed in tissues outside the nervous system. It has been confirmed that Msi-1 is preferentially expressed in the predicted stem cell regions of mouse and human intestinal crypts, suggesting that it can serve as a potential marker for intestinal stem/progenitor cells<sup>[5,6]</sup>. Msi-1 has also been observed in the stomach of chickens<sup>[7]</sup>, mice<sup>[7]</sup>, rats<sup>[8]</sup> and humans<sup>[9]</sup>. Akasaka *et al*<sup>[9]</sup> reported that Msi-1 is mostly expressed in the antrum and Murata *et al*<sup>[10]</sup> found that it is expressed in both the antrum and corpus of the human stomach. For the past several years, the functional role of Msi-1 in tumors has attracted increasing interest. Msi-1 overexpression has been reported in tumor tissues

and cell lines, such as medulloblastoma<sup>[4]</sup>, astrocytoma<sup>[11]</sup>, retinoblastoma<sup>[12]</sup> and endometrial carcinoma<sup>[13]</sup>.

Recently, Wang *et al*<sup>[14]</sup> selected 10 cases of intestinal-type GC taken from the transitional area between malignancy and adjacent normal mucosa for full section analysis. They found that Msi-1 was frequently expressed in both premalignant gastric lesions and invasive GC; however, the number of patients with premalignant gastric lesions in this study was low. Proliferating cell nuclear antigen (PCNA) is the auxiliary protein of DNA polymerase  $\delta$  and can be used as a good indicator of GC cell proliferation and prognosis<sup>[15]</sup>. In the present study, to explore proliferation activity diversity of Msi-1-positive cells in the development of GC, we investigated the expression of Msi-1 and PCNA in intestinal-type GC and precancerous lesions, including 41 high-grade intraepithelial neoplasia, 57 low-grade intraepithelial neoplasia, and 31 intestinal metaplasia. The correlation between Msi-1 expression and various clinicopathological parameters was also studied. Additionally, we aimed to determine whether Msi-1 is a candidate marker of intestinal-type GC stem cells because this would be an important aspect of the function of Msi-1 in gastric carcinogenesis.

## MATERIALS AND METHODS

### *Clinical and pathological features*

Endoscopic biopsy antral specimens were obtained from September 2008 to January 2011 at Qianfoshan Hospital Affiliated to Shandong University, including: 36 cases of normal gastric mucosa (21 males and 15 females; aged 39-60 years, mean  $57 \pm 7.2$  years); 31 cases of intestinal metaplasia (17 males and 14 females; aged 43-70 years, mean  $54 \pm 8.4$  years); 57 cases of low-grade intraepithelial neoplasia (33 males and 24 females; aged 45-79 years, mean  $58 \pm 8.2$  years); and 41 cases of high-grade intraepithelial neoplasia (23 males and 18 females; aged 46-80 years, mean  $57 \pm 9.6$  years). The other 54 cases were intestinal-type GC, including 29 males and 25 females, aged 40-72 years, mean age  $57 \pm 9.5$  years. All the pathological specimens were diagnosed and reviewed by three veteran pathologists.

According to tumor node metastasis (TNM) stage of the Union for International Cancer Control in 1997, GC is divided into I a, I b, II, III a, III b and IV stage. Twenty-seven cases of I a and I b stage were considered as the early clinical GC (I stage), including 17 male and 10 female patients, with a mean age of  $56 \pm 9.4$  years. Twenty-seven cases of II-IV stage GC merged in the late stage GC (II-IV stage), including 12 male and 15 female patients, with a mean age of  $57 \pm 9.8$  years. On the basis of the depth of cancer invasion, 20 cases were limited to mucosa and submucosa ( $\leq T1$ ) and 34 cases broke through the submucosa (T2-4). In GC metastasis, there were 27 patients with lymph node metastasis and 27 without, or 14 patients with distant metastasis and 40 without. No significant difference in age and sex existed among all the groups of patients.

### Immunohistochemistry

Specimens were fixed in 10% paraformaldehyde, conventionally dehydrated, embedded in paraffin, and sliced into 4- $\mu$ m-thick serial sections. The microwave ethylene diamine tetraacetic acid (EDTA) (pH 9.0) antigen retrieval method was used to retrieve the antigen. A two-step immunohistochemical method was used to observe the antigen expression. Rabbit polyclonal anti-human Msi-1 antibody (ab52685; 1:300 dilution) and mouse anti-human PCNA monoclonal antibody (ab29; 1:1000 dilution) were purchased from Abcam Company (Cambridge, MA, United States).

### Assessment of cell staining

Cells were considered positive for Msi-1 when the cytoplasm contained evenly stained yellow or brown granules. PCNA positivity was adjudged by the appearance of brown or yellow granules in the nucleus. Tissue sections were observed microscopically at  $\times 400$  magnification. Five representative regions were selected with 200 cells each. Msi-1 staining intensity was defined as follows: no staining or  $< 10\%$  of cells stained, negative (-); 10%-30% stained cells, weakly positive (+); 31%-50% stained cells, positive (++); and  $> 50\%$  stained cells, strongly positive (+++). PCNA staining intensity was defined as follows: no staining or  $< 25\%$  of cells stained, negative (-); 25%-50% stained cells, weakly positive (+); 51%-75% stained cells, positive (++); and  $> 75\%$  stained cells, strongly positive (+++).

### Statistical analysis

All statistical analyses were performed using SPSS version 13.0. Measurement data were analyzed using Student's *t* test and categorical data were studied using the  $\chi^2$  test, Fisher's exact test or nonparametric rank sum test. The Spearman correlation test was used to determine the correlation between expression of Msi-1 and PCNA. Correlations between Msi-1 expression and clinicopathological parameters were also statistically analyzed. Statistical significance was taken as  $P < 0.05$ .

## RESULTS

### Expression of Msi-1 and PCNA in different pathological tissues

There were significant differences in Msi-1 and PCNA expression in different pathological tissues ( $\chi^2 = 15.37$ ,  $P < 0.01$ ;  $\chi^2 = 115.36$ ,  $P < 0.01$ ). Msi-1 and PCNA-positive cells were restricted to the isthmus of gastric glands in normal gastric mucosa (Figures 1A and 2A). The expression levels of Msi-1 and PCNA in intestinal metaplasia were higher than in normal mucosa ( $U = 392.0$ ,  $P < 0.05$ ;  $U = 40.50$ ,  $P < 0.01$ ) and the distribution of Msi-1 and PCNA-positive cells was more diffuse (Figures 1B and 2B). Expression of Msi-1 and PCNA showed no significant difference among intestinal metaplasia, low and high-grade intraepithelial neoplasia (Figures 1C, 1D, 2C and 2D) ( $\chi^2 = 2.72$ ,  $P > 0.05$ ;  $\chi^2 = 1.64$ ,  $P > 0.05$ ). Msi-1

expression in intestinal-type GC was higher than in high-grade intraepithelial neoplasia (Figure 1E) ( $U = 798.0$ ,  $P < 0.05$ ) and PCNA expression in GC was also higher than in high-grade intraepithelial neoplasia (Figure 2E) ( $U = 688.0$ ,  $P < 0.01$ ) (Table 1).

### Relationship between Msi-1 expression and GC invasion and metastasis

No significant difference existed in Msi-1 expression between tumor invasion depth  $\leq T1$  and T2-4 ( $\chi^2 = 7.37$ ,  $P > 0.05$ ). Msi-1 expression was higher in GC with than without lymph node metastasis ( $\chi^2 = 12.62$ ,  $P < 0.01$ ). There was no significant difference between Msi-1 expression in GC with and without distant metastasis ( $\chi^2 = 7.06$ ,  $P > 0.05$ ). Msi-1 expression was higher in GC II - IV stage group than in stage I group ( $\chi^2 = 11.24$ ,  $P < 0.05$ ) (Figure 3, Table 2).

### Correlation between expression of Msi-1 and PCNA

Spearman's bivariate correlation analysis was made between Msi-1 and PCNA expression in different pathological tissues. Msi-1 and PCNA expression showed a significant positive correlation ( $r = 0.20$ ,  $P < 0.01$ ) (Table 3).

## DISCUSSION

The epithelial stem cells of the adult stomach are thought to be present in the proliferative region of the isthmus of the gastric glands. They constantly regenerate and migrate bidirectionally, up to the mucosal surface and down to the gland base, as they differentiate into mature cells of the gastric unit<sup>[16]</sup>. In the present study, immunohistochemistry demonstrated the presence of small numbers of PCNA-positive cells concentrated in the isthmus region of normal gastric glands, suggesting that this region is the proliferative zone of the gastric glands. We found that Msi-1 expression was also limited to the isthmus region of the gastric glands where adult putative stem cells are located. The positive correlation between the distribution of Msi-1-positive cells and that of the proliferative regions in the gastric glands suggests that Msi-1-positive cells have stem cell characteristics in the human stomach. Akasaka *et al.*<sup>[9]</sup> have reported a similar expression pattern of Msi-1 in the human antrum, suggesting that Msi-1 can be useful for identifying stem cells in the human stomach.

Our observations indicate that Msi-1 expression can correlate positively with proliferative activity in the stomach, but the correlation coefficient is low. All the cells in the gastrointestinal epithelium are renewed within a short time; thus, it is possible that precursor cells, not stem cells, in the proliferative zone produce a large number of mature cells within a short time. Indeed, it has been proposed that the proliferative zone has a pyramid-like structure, consisting of various cell layers with stem cells on top, and that the number of stem cells produced is small<sup>[17]</sup>. In our study, PCNA expression showed proliferative activity in all the cells in the gastric tissue. We be-

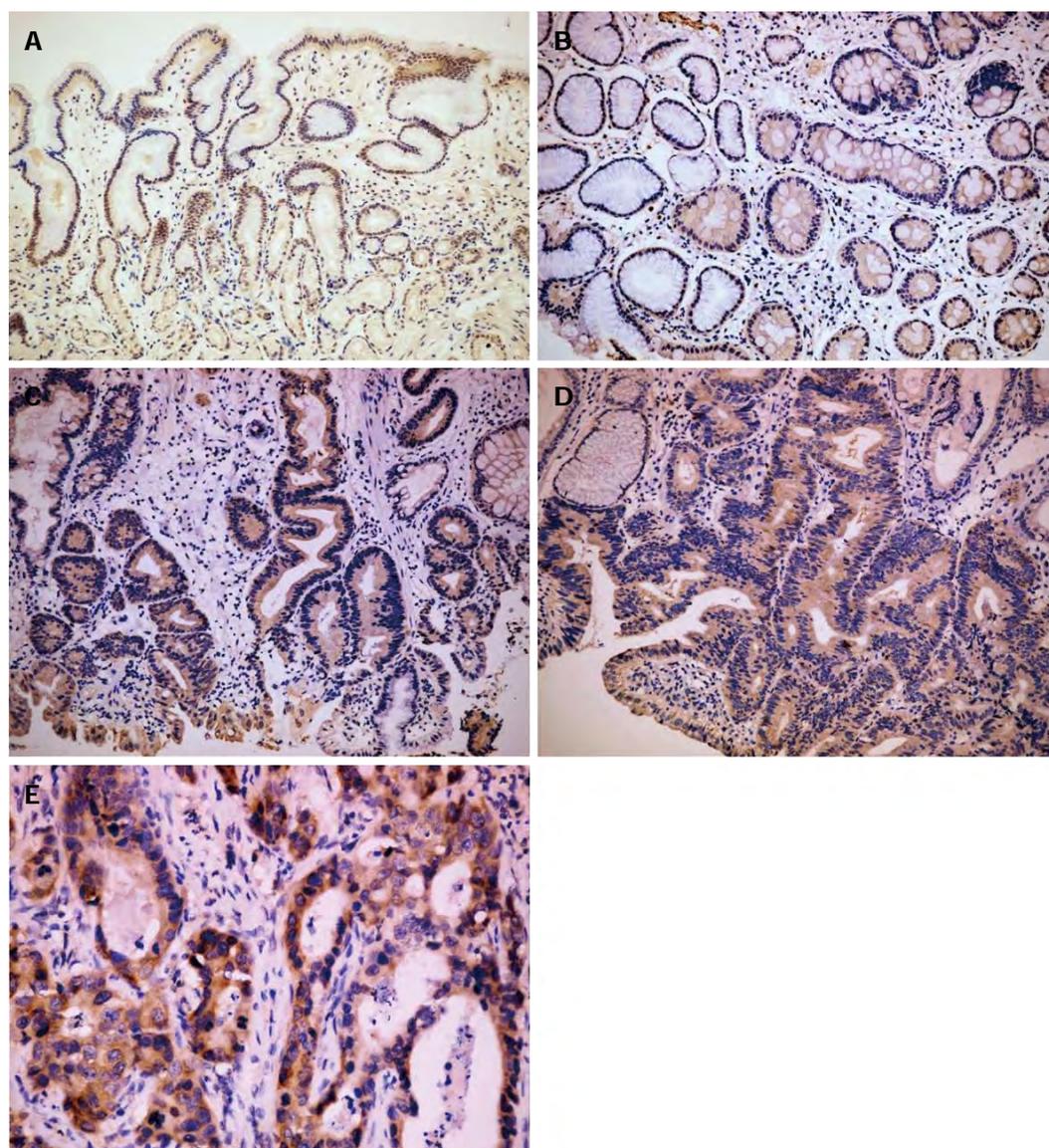


Figure 1 Immunohistochemical staining for Musashi-1 in different gastric tissues. A: A weak expression of Musashi-1 (Msi-1) was observed in the isthmus of normal gastric glands ( $\times 100$ ); B: The expression of Msi-1 was significantly increased in intestinal metaplastic mucosa ( $\times 100$ ); C: Msi-1 expression showed no significant difference between in low grade intraepithelial neoplasia ( $\times 100$ ); D: high grade intraepithelial neoplasia ( $\times 100$ ); E: The expression of Msi-1 was increased again in the intestinal type gastric cancer ( $\times 400$ ).

Table 1 Expression intensity of Musashi-1 and proliferating cell nuclear antigen in different pathological tissues

Pathological tissues	Msi-1 protein staining intensity (n)				PCNA protein staining intensity (n)			
	-	+	++	+++	-	+	++	+++
Normal gastric mucosa	17	19	0	0	27	9	0	0
Intestinal metaplasia <sup>a,b</sup>	7	21	3	0	0	9	10	12
Low grade intraepithelial neoplasia	21	32	4	0	0	9	29	19
High grade intraepithelial neoplasia	19	17	4	1	0	6	16	19
Intestinal-type GC <sup>c,d</sup>	12	29	9	4	0	0	10	44

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , significantly different *vs* normal gastric mucosa; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , significantly different *vs* high-grade intraepithelial neoplasia. PCNA: Proliferating cell nuclear antigen; Msi-1: Musashi-1; GC: Gastric cancer.

lieve that the expression of Msi-1 in the human stomach is associated with cell proliferation; however, because the number of stem cells is small, the correlation is not close.

Intestinal metaplasia is the precancerous lesion of

intestinal-type GC<sup>[18]</sup>. Akasaka *et al*<sup>[9]</sup> reported that Msi-1 expression was markedly decreased in intestinal metaplasia cells of the human stomach. They believe that intestinal metaplasia is a consequence of abnormal differentia-

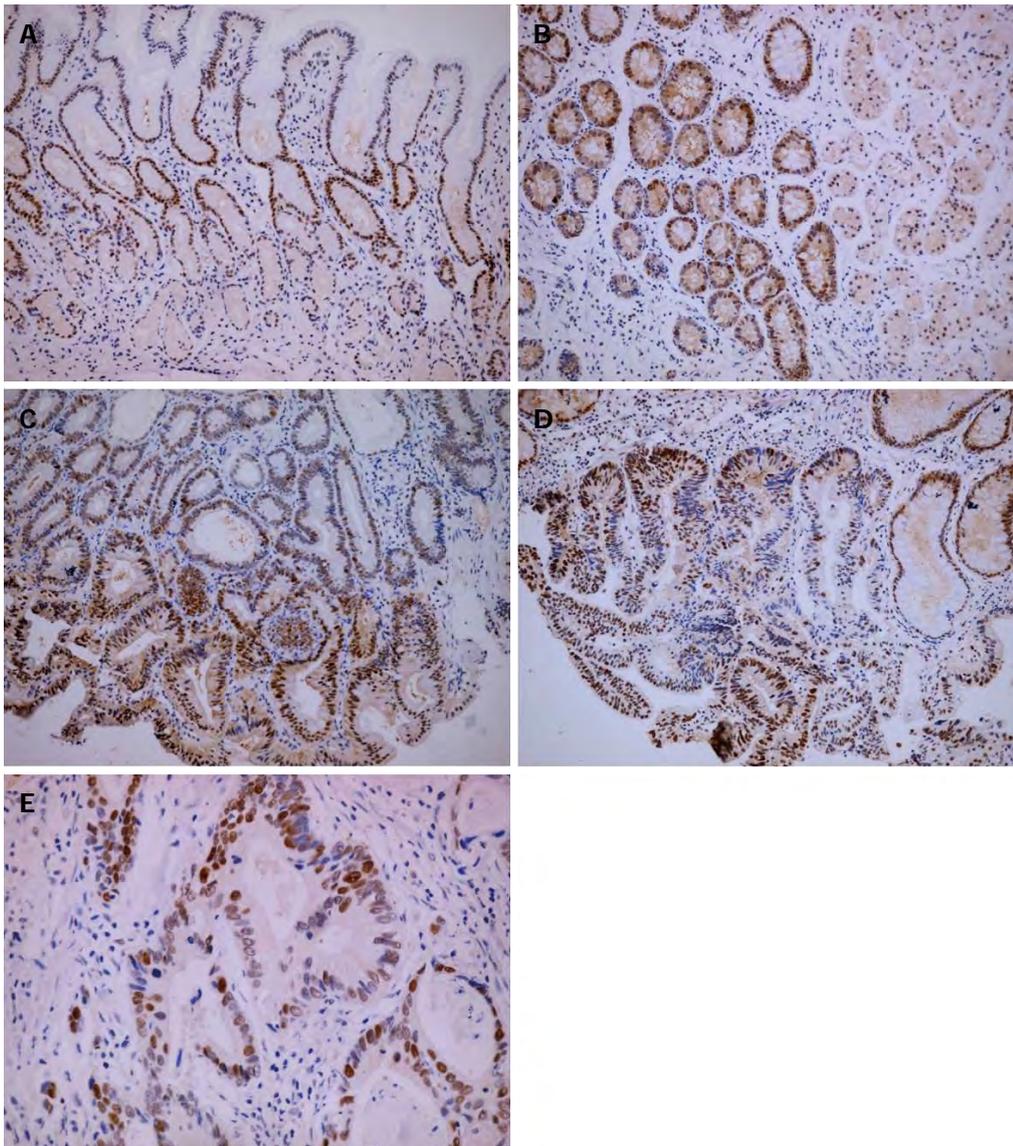


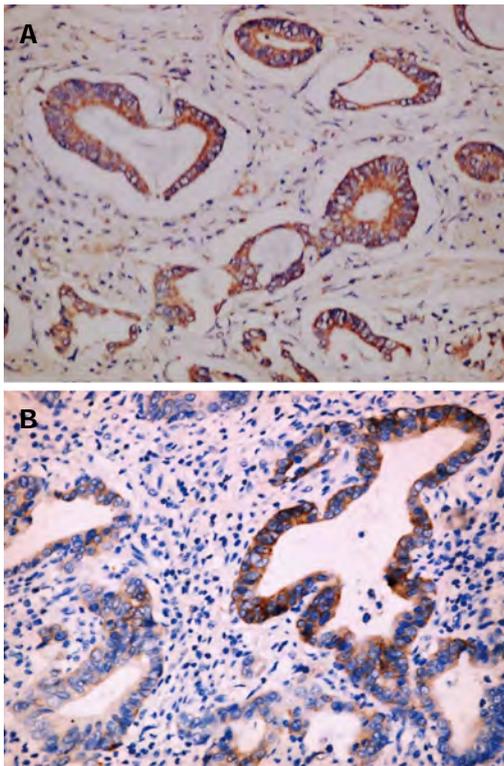
Figure 2 Immunohistochemical staining for proliferating cell nuclear antigen was increased along with the development of gastric carcinogenesis. A: The staining of proliferating cell nuclear antigen (PCNA) in the isthmus of normal gastric glands ( $\times 100$ ); B: Intestinal metaplastic mucosa ( $\times 100$ ); C: Low grade intraepithelial neoplasia ( $\times 100$ ); D: High grade intraepithelial neoplasia ( $\times 100$ ); E: Strong staining of PCNA in intestinal type gastric cancer ( $\times 400$ ).

**Table 2 Relationship between Musashi-1 expression and clinicopathological parameters**

Variable	Group	No.	Msi-1 staining intensity			
			-	+	++	+++
Infiltration depth	$\leq T1$	20	8	9	3	0
	T2-4	34	4	20	6	4
Lymph node metastasis <sup>a</sup>	NO	27	11	9	5	2
	YES	27	1	20	4	2
Distant metastasis	NO	40	12	21	5	2
	YES	14	0	8	4	2
TNM staging <sup>b</sup>	Stage I	27	11	10	4	2
	Stage II-IV	27	1	19	5	2

There was a significant difference between Musashi-1 (Msi-1) expression and clinicopathological parameters in Gastric cancer. <sup>a</sup> $P < 0.01$  and <sup>b</sup> $P < 0.05$ . TNM: Tumor node metastasis.

tion of stem cells, in which tissue-specific stem cells in the stomach may not differentiate into any of the normal intestinal epithelial phenotypes. In our study, however, more Msi-1-positive cells were observed in the intestinal metaplasia than in the normal gastric mucosa and the distribution was also more diffuse. It is known that Msi-1 is not a tissue-specific marker of epithelial stem cells. We believe that Msi-1 can be expressed in epithelial stem cells in both gastric mucosa and intestinal metaplasia, but proliferation of stem cells is different, which may be due to the mode of fission. In normally growing gastric tissues, stem cells undergo a slow but constant self-renewal by asymmetric division and give rise to all the various gastric epithelial cell types by differentiation<sup>[19]</sup>. These properties are essential to their normal role in gastric tissues because



**Figure 3** Musashi-1 expresses higher in intestinal type gastric cancer classified as stage II-IV. A: Type gastric cancer classified as stage II-IV; B: Those classified as stage I ( $\times 400$ ).

stem cells maintain tissue homeostasis by regulating cell turnover, depending on the demand at any given time. Surprisingly, experimental and mathematical modeling studies have indicated that intestinal crypt stem cells divide symmetrically and stochastically, and not with the asymmetry observed in stem cell division in some other tissues<sup>[20,21]</sup>. Similarly, symmetric division among intestinal metaplasia stem cells probably leads to accelerated division of stem cells, adapting to high proliferative activity. Murata *et al*<sup>[10]</sup> demonstrated that Msi-1 was expressed in gastric glands with intestinal metaplasia where proliferative activity was relatively high. Similar results were also obtained in the study of Wang *et al*<sup>[14]</sup>. Therefore, by influencing dividing cells, Msi-1 might regulate metaplastic changes.

We found that there was no significant difference between intestinal metaplasia and low and high-grade intraepithelial neoplasia for the expression of PCNA and Msi-1, suggesting that there was similar proliferation of epithelial stem cells in the gastric precancerous lesions. An *in vitro* study in primary cultures of intestinal epithelium showed that Msi-1 overexpression promotes the proliferation of stem cells and activates Wnt and Notch pathways. Moreover, Msi-1-overexpressing cells exhibited tumorigenic properties in xenograft experiments<sup>[22]</sup>. Using mitochondrial DNA mutations as a marker of clonal expansion, McDonald *et al*<sup>[23]</sup> investigated how mutations expand in gastric mucosa showing signs of intestinal metaplasia. They have shown that intestinal metaplastic crypts are

**Table 3** Relationship between Musashi-1 and proliferating cell nuclear antigen expression

Msi-1 protein staining intensity	PCNA protein staining intensity				Total
	Negative	Weak positive	Positive	Strong positive	
Negative	10	23	17	26	76
Weak positive	17	7	40	54	118
Positive	0	2	7	11	20
Strong positive	0	1	1	3	5
Total	27	33	65	94	219

PCNA: Proliferating cell nuclear antigen; Msi-1: Musashi-1.

clonal, possess multiple stem cells, and that fission is a mechanism by which intestinal metaplasia spreads. The expansion and spread of mutated gastric stem cells is one mechanism of carcinogenesis in the human stomach. Our findings suggest that expansion of Msi-1-positive cells in the intestinal metaplasia mucosa may be an early event in initiating the intestinal metaplasia - intraepithelial neoplasia - adenocarcinoma cascade. Stem cell amplification caused by deregulation of this self-renewal process may play a key role in the early events of gastric carcinogenesis.

We found that there were significantly more PCNA-positive and Msi-1 positive cells in intestinal-type GC than in gastric precancerous lesions, suggesting that Msi-1 could be used as a potential marker for intestinal-type GC stem cells, and that cancer stem cells have stronger proliferation compared to stem cells in precancerous lesions. Cancer stem cells are able to sustain and propagate tumors and give rise to invasive lesions and metastases<sup>[24]</sup>. In agreement with our data, Msi-1-positive cells may be involved in tumor invasion and metastasis because Msi-1 expression correlated with lymph node metastasis and TNM stage of GC. Msi-1 plays a fundamental role in maintaining stem cells in an undifferentiated state and it has been implicated in proliferative pathology in various tissues where Msi-1 may be acting to promote self-renewal of tumor cells with stem cell-like properties<sup>[25-27]</sup>. Small cell pulmonary carcinoma is usually an aggressive neoplasm with high metastatic potential and recurrence. Moreira *et al*<sup>[28]</sup> reported that small cell carcinomas demonstrate diffuse expression of Msi-1, whereas focal or isolated positivity is seen in most other types of pulmonary carcinoma. In human colorectal cancer, Msi-1 protein expression is significantly higher in tissue samples classified as stage III than stage I or II<sup>[29]</sup>. It was demonstrated that siRNA-mediated knockdown of Msi-1 in the HCT116 colon adenocarcinoma xenografts resulted in the arrest of tumor growth<sup>[30]</sup>. Knockdown of Msi-1 expression by siRNA induced apoptosis and a severe decline in cell numbers in 5637 bladder carcinoma cells<sup>[31]</sup>. These results suggest that Msi-1 plays an important role in tumorigenesis and tumor progression. We speculate that Msi-1 overexpression contributes to maintain cancer stem cells in an undifferentiated state, increasing their capacity for self-renewal or proliferation, thus promoting GC invasion and metastasis.

Shen *et al.*<sup>[32]</sup> proposed that cancer may arise from a long development process of tumor-initiating cells - precancerous stem cells - cancer stem cells - cancer, which is in parallel to histological changes of hyperplasia - precancer - carcinoma, accompanied by clonal evolutionary epigenetic and genetic alterations. In the present study, we demonstrated that expansion of Msi-1-positive cells appeared to be an early event in the process of gastric carcinogenesis and proliferation of Msi-1-positive cells was different in GC and precancerous lesions. The precancerous stem cells, representing the early stage of developing cancer stem cells, have the potential for both benign and malignant differentiation, depending on environmental cues<sup>[33]</sup>. Therefore, a broad therapeutic approach to GC may be achievable through rational targeting of precancerous stem cells. More work is needed to investigate the differentiation of Msi-1-positive cells between GC and precancerous lesions. However, Msi-1 may eventually represent a biomarker that can be used for early diagnosis of GC or to monitor cancer progression and response to therapy.

## COMMENTS

### Background

Gastric cancer (GC) remains one of the leading causes of global cancer mortality. The gastric epithelium is continuously regenerated by gastric stem cells, which give rise to various kinds of epithelial cells. GC is currently thought to be a disease originating in stem cells and it is hypothesized that cancer stem cells drive cancer growth and metastasis. However, the lack of useful markers has made it difficult to characterize the stem cells in the human gastric mucosa and has hindered study of the origin of GC. The RNA binding protein Musashi-1 (Msi-1) is a putative stem cell marker. However, the role of Msi-1 in GC and precancerous lesions is not fully understood.

### Research frontiers

Msi-1, a neural RNA-binding protein, was initially identified as a neuronal stem cell marker. In recent years, Msi-1 has been found in tissues outside the nervous system and identified as a putative intestinal and gastric stem cell marker. For the past several years, overexpression of Msi-1 has been reported in many tumor tissues and cell lines. High levels of Msi-1 expression in glioma and astrocytoma indicate a poor prognosis. Knockdown of Msi-1 in the HCT116 colon adenocarcinoma xenografts resulted in the arrest of tumor growth. These results suggest that Msi-1 plays an important role in tumorigenesis and tumor progression.

### Innovations and breakthroughs

Msi-1 has been proposed as a putative stem cell marker in the mouse intestine and human stomach but little is known about the role of such markers in the overall pathway of gastric carcinogenesis. Moreover, the results of previous studies in intestinal metaplasia are different. In this study, the authors explored proliferation diversity of Msi-1-positive cells in the development of GC and found that the expression levels of Msi-1 and proliferating cell nuclear antigen (PCNA) in intestinal metaplasia were significantly higher than in normal mucosa, while there were no significant differences compared with intraepithelial neoplasia. Msi-1 and PCNA expression in intestinal-type GC was higher than in intraepithelial neoplasia. Msi-1 expression in GC tissues correlated with their lymph node metastasis and TNM stage. There was a significantly positive relationship between Msi-1 and PCNA expression. These results suggest that Msi-1-positive cells may play a key role in the early events of gastric carcinogenesis, and may be involved in invasion and metastasis of GC.

### Applications

The study results contribute to a better understanding of the associations between putative stem cell marker expression and tumorigenesis and progression of GC. The knowledge gained by studying cancer stem cells in gastric mucosa will support the development of novel therapeutic strategies for GC. Msi-1 may

eventually represent a biomarker that can be used for early diagnosis of GC or to monitor cancer progression and response to therapy.

### Terminology

Cancer stem cells are defined as the unique subpopulation in tumors that possess the ability to initiate tumor growth and sustain self-renewal, as well as metastatic potential. Musashi is an evolutionarily conserved family of RNA-binding proteins that is preferentially expressed in the nervous system. The first member of the Musashi family was identified in *Drosophila*. Its mammalian homolog, Msi-1, is a neural RNA-binding protein that is selectively expressed in murine and human neural stem cells, can be used as a neural stem cell marker, and plays important roles in the maintenance of stem cell states, as well as in the regulation of differentiation of stem cells.

### Peer review

The study was well designed and performed. The authors detected stem cell marker Msi-1 and PCNA expression in the multistep process of gastric carcinogenesis. The results are interesting and suggest that the expansion of Msi-1-positive cells is an early event in the process of gastric carcinogenesis and may be involved in invasion and metastasis of GC.

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## Can eradication rate of gastric *Helicobacter pylori* be improved by killing oral *Helicobacter pylori*?

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### Abstract

**AIM:** To evaluate the influence of oral *Helicobacter pylori* (*H. pylori*) on the success of eradication therapy against gastric *H. pylori*.

**METHODS:** A total of 391 patients with dyspepsia were examined for *H. pylori* using the saliva *H. pylori* antigen test (HPS), <sup>13</sup>C-urea breath test (UBT), gastroscopy, and gastric mucosal histopathological detection. Another 40 volunteers without discomfort were subjected to HPS and <sup>13</sup>C-UBT, and served as the control group. The 233 patients who were <sup>13</sup>C-UBT+ were enrolled in this study and divided into 4 groups. Patients who were HPS- and <sup>13</sup>C-UBT+ (*n* = 53) received triple therapy alone. Those who were both HPS+ and <sup>13</sup>C-UBT+ (*n* = 180) were randomly divided into 3 groups: (1) the O+G+t group which received triple therapy alone (*n* = 53); (2) the O+G+tm group which received both triple therapy and mouthrinse treatment (*n* = 65); and (3) the O+G+tmp group which received triple therapy, mouthrinse, and periodontal treatment (*n* = 62). The HPS and <sup>13</sup>C-UBT were continued for 4 wk after completion of treatment, and the eradication rate of gastric *H. pylori* and the prevalence of oral *H. pylori* in

the 4 groups were then compared.

**RESULTS:** The eradication rates of gastric *H. pylori* in the O-G+t group, the O+G+tm group, and the O+G+tmp group were 93.3%, 90.0%, and 94.7% respectively; all of these rates were higher than that of the O+G+t group (78.4%) [O-G+t group vs O+G+t group (*P* = 0.039); O+G+tm group vs O+G+t group (*P* = 0.092); O+G+tmp group vs O+G+t group (*P* = 0.012); O+G+tm group vs O-G+t group (*P* = 0.546); O+G+tmp group vs O-G+t group (*P* = 0.765); O+G+tm group vs O+G+tmp group (*P* = 0.924)]. The eradication of gastric *H. pylori* was significantly improved using the combination of triple therapy, mouthrinse, and periodontal treatment. The eradication rates of gastric *H. pylori* in the peptic ulcer group, chronic atrophic gastritis group and control group were higher than in the duodenitis group and the superficial gastritis group. The prevalence rates of oral *H. pylori* in the O-G+t group, O+G+t group, O+G+tm group and O+G+tmp group following treatment were 0%, 76.5%, 53.3%, and 50.9%, respectively [O-G+t group vs O+G+t group (*P* < 0.0001); O+G+tm group vs O+G+t group (*P* = 0.011); O+G+tmp group vs O+G+t group (*P* = 0.006); O+G+tm group vs O-G+t group (*P* < 0.0001); O+G+tmp group vs O-G+t group (*P* < 0.0001); O+G+tm group vs the O+G+tmp group (*P* = 0.790)]. Both mouthrinse and periodontal treatment significantly reduced the prevalence of oral *H. pylori*.

**CONCLUSION:** Mouthrinse treatment alone or combined with periodontal treatment can, to some extent, reduce the prevalence of oral *H. pylori* and improve the eradication rate of gastric *H. pylori*.

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**Key words:** *Helicobacter pylori*; Dental plaque; Eradication; Periodontal; Mouthrinse

**Core tip:** The average eradication rate of gastric *Helico-*

*bacter pylori* (*H. pylori*) has decreased in recent years. However, some foreign studies have shown that the eradication rate of gastric *H. pylori* may be improved by eliminating the presence of oral *H. pylori* rather than increasing the dose of antibiotics. In most studies, *H. pylori* DNA was detected and used to confirm oral *H. pylori* infection, and later determine whether professional periodontal treatments were effective in killing oral *H. pylori*. To avoid the expensive and complicated techniques involved with this approach, the current study used a cost-effective and simple method to test for and eliminate oral *H. pylori*. This method can be used to prove the elimination of gastric *H. pylori*, and is practical for use in the clinic.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is believed to be one of the major factors responsible for chronic active gastritis, gastroduodenal ulcers, mucosa-associated lymphoid tissue lymphomas, and gastric cancers<sup>[1]</sup>. It was designated as a type I carcinogen by the World Health Organization in 1994 and approximately 50% of the world's population is infected. The isolation of *H. pylori* from dental plaque by Krajde<sup>[2]</sup> in 1989 strongly suggested that both oral and gastro-oral routes are important transmission modes of *H. pylori*, and that the oral cavity is an extra-gastric reservoir for *H. pylori*<sup>[3-6]</sup>. Several studies have suggested that oral *H. pylori* is associated with the presence of gastric *H. pylori*<sup>[6,7]</sup>; additionally, patients who are oral *H. pylori*-positive have a lower success rate of gastric *H. pylori* eradication than patients who test negative for oral *H. pylori*<sup>[6,8,9]</sup>. Also, previous studies have shown that DNA samples obtained from oral *H. pylori* are very similar to those obtained from corresponding gastric *H. pylori*<sup>[10-12]</sup>. The prevalence of oral *H. pylori* is related to an individual's quality of oral hygiene and periodontal status, such as the presence of dental plaque and periodontal pockets<sup>[13]</sup>, and the eradication rate of gastric *H. pylori* can be increased by controlling dental plaque and improving oral hygiene<sup>[14,15]</sup>. In our study, the saliva *H. pylori* antigen test (HPS) and the <sup>13</sup>C-urea breath test (<sup>13</sup>C-UBT) were used to detect oral and gastric *H. pylori* infections, respectively, and in this report, the term HPS+ signifies the presence of oral *H. pylori* infection, while <sup>13</sup>C-UBT+ signifies the presence of gastric *H. pylori* infection. <sup>13</sup>C-UBT+ patients were recruited for this study. HPS- cases were treated with triple therapy alone, while HPS+ cases were randomly distributed into 3 groups which received different treatments. The goal of this study was to evaluate the influence of oral *H. pylori* on the success of eradication

therapy against gastric *H. pylori*.

## MATERIALS AND METHODS

### Patient population

From August 2011 to July 2012, outpatients with dyspepsia seen at the Department of Gastroenterology, Shengjing Hospital of China Medical University, Shenyang, China, were recruited and selected for this study. Exclusion criteria included a past history of *H. pylori* eradication therapy; treatment with antibiotics, H<sub>2</sub> receptor blockers, bismuth or proton pump inhibitors within one month of study enrollment; the presence of severe periodontal disease; presence of an immune disease; current pregnancy; age < 18 years; and the use of immune depressant drugs. A total of 391 eligible patients were enrolled in the study and another 40 volunteers without discomfort were enrolled to serve as a control group (Table 1).

The diseases listed in Table 1 were diagnosed using the following criteria: Peptic ulcer was defined as the presence of gastric ulcer and/or duodenal ulcer. Chronic atrophic gastritis: Endoscopy showed good visualization of the submucosal vessel in the antrum and in the body. Histopathology showed the loss of appropriate glands or the presence of metaplasia. Duodenitis: During endoscopy, the duodenal bulb mucosa appeared abnormally congested, edematous, or roughened in the absence of an ulcer or scar. Histopathology showed nuclear atypia of the glandular epithelium and infiltration by neutrophils. Superficial gastritis: During endoscopy, gastric mucosa appeared abnormally congested, edematous, or roughened. Histopathology showed nuclear atypia of the glandular epithelium and infiltration by neutrophils.

### Study groups

Each subject was evaluated by HPS, <sup>13</sup>C-UBT, gastroscopy, and gastric mucosal histopathological examination. Two biopsy specimens were taken from the greater curvature of both the antrum and the body of the stomach, respectively. Another 2 biopsy specimens were taken from both the lesser curvature of the antrum and body, respectively. Patients who were HPS- and <sup>13</sup>C-UBT+ (*n* = 53) received triple therapy alone. Those who were both HPS+ and <sup>13</sup>C-UBT+ (*n* = 180) were randomly divided into 3 groups: (1) the O+G+t group which received triple therapy alone (*n* = 53); (2) the O+G+tm group which received triple therapy and mouthrinse treatment (*n* = 65); and (3) the O+G+tmp group which received triple therapy, mouthrinse, and periodontal treatment (*n* = 62). The triple therapy consisted of amoxicillin (1.0 g) and esomeprazole (20 mg), twice a day, and levofloxacin (0.5 g), once a day, given for 10 d. Mouthrinse (brand name Chlorhexidine Gargle), (Shenzhen Nanyue Pharmaceutical Co., Ltd., Shenzhen, China) was purchased from a drugstore. This mouthwash contained 0.02% tinidazole and 0.12% chlorhexidine, and a 20 mL volume was held in the mouth for 5 min and then spat out, for 10 d. Periodontal therapy consisted of plaque and calculus removal with an ultrasonic device twice a month in the Depart-

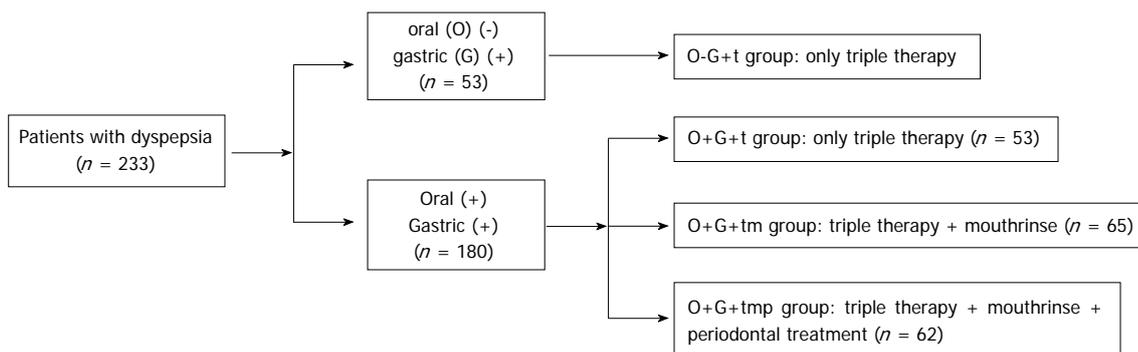


Figure 1 Study design schematic and study measures.

**Table 1 Characteristics of the 431 patients**

Diseases	Total	Male	Female	Age range (yr)	Mean age (yr)
Peptic ulcer	54	40	14	20-80	51.1
Chronic atrophic gastritis	48	32	16	46-82	58.5
Duodenitis	58	31	27	26-77	51.0
Superficial gastritis	231	95	136	18-74	49.6
Control group	40	17	23	18-67	44.0

ment of Stomatology, Shengjing Hospital of China Medical University. HPS and <sup>13</sup>C-UBT testing were conducted 4 wk after triple therapy was completed. A flow-chart of patient treatment is shown in Figure 1.

**HPS**

The saliva *H. pylori* antigen test (Meili Taige Diagnostic Reagent Co., Ltd, Jiaxing, China) is a rapid immunochromatographic assay that uses antibody-coated colloidal gold to detect the presence of specific *H. pylori* antigens in the saliva specimen. Yee *et al*<sup>[16]</sup> clarified that HPS was designed to detect two *H. pylori* antigens: flagellin and urease. In Yee’s experiment, the HPS results were compared in parallel with the UBT, serum antibody, Campylobacter-like organism test, silver stain, culture, and stool antigen test results. The test’s sensitivity was 10 ng/mL *H. pylori* flagellin and urease antigen. There was no interference or cross-reactivity with the other bacteria in the oral cavity and there was statistical correlation between oral antigen and serum antibody test results. No food or drink was allowed 1 h before the test. To perform the test, four drops of saliva were added to the sampling cup using a pipette and two drops of PBS were added. After mixing, a new pipette was used to transfer four drops of the mixture into the sample well of the test cassette. The sample flowed through a label pad containing *H. pylori* antibody coupled to red-colored colloidal gold. If the sample contained *H. pylori* antigens, the antigen would bind to the antibody coated on the colloidal gold particles to form antigen-antibody-gold complexes. The complexes then moved on a nitrocellulose membrane by capillary action toward the test zone. A second control line always appeared in the result window to indicate that the test had been correctly performed and that the test device was functioning prop-

erly. The results were observed within 5-30 min. The occurrence of two bands in the test and control zones was positive for *H. pylori*. The occurrence of one band in the control zone was negative for *H. pylori*. If there was no band in the control zone, the samples were re-tested.

**<sup>13</sup>C-UBT**

An HG-IRIS<sup>13</sup>C infrared spectrometer and diagnostic kits (Beijing Pharmaceutical Co., Ltd) were used to detect gastric *H. pylori*. The test was judged positive when the detected value in the exhalation was larger than 4.

**Statistical analysis**

Statistical analysis of data was performed with SPSS software 19.0. The  $\chi^2$  test was used to analyze the eradication rate of gastric *H. pylori* and the prevalence of oral *H. pylori* in each group. A *P* value  $\leq 0.05$  was considered statistically significant.

**RESULTS**

**Results of HPS and <sup>13</sup>C-UBT testing**

A total of 431 patients were tested using HPS and <sup>13</sup>C-UBT methods, and the results are shown in Table 2.

The prevalence of gastric and oral *H. pylori* differed among the 5 disease groups. There was a reduced trend for the prevalence of gastric *H. pylori* starting from the peptic ulcer group and continuing to the control group, but no similar trend was seen for oral *H. pylori* (Figure 2).

**Eradication rate of gastric *H. pylori* after treatment**

Four weeks after completion of treatment, 213 patients returned for evaluation, and 20 patients did not return. The gastric *H. pylori* eradication rate in the O+G+t group was significantly lower than in the O-G+t and O+G+tmp groups (O-G+t group *vs* O+G+t group, *P* = 0.039), (O+G+tmp group *vs* O+G+t group, *P* = 0.012). The gastric *H. pylori* eradication rate in the O+G+tm group was higher than in the O+G+t group, but the difference was not statistically significant (*P* = 0.092). There were no statistical differences between the O-G+t and O+G+tm groups when compared to the O+G+tmp group (O+G+tm group *vs* O-G+t group, *P* = 0.546), O+G+tmp group *vs* O-G+t group, *P* = 0.765), and

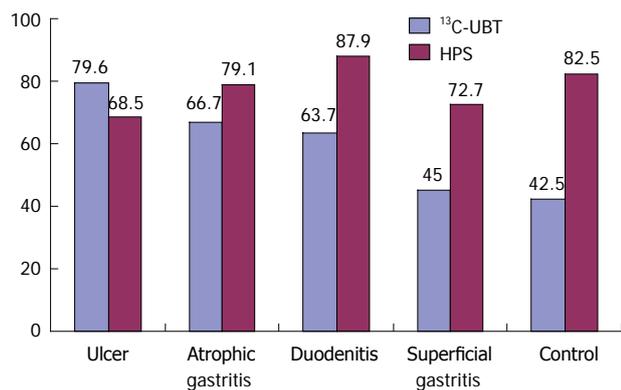


Figure 2 Percentages of positive <sup>13</sup>C-urea breath test and *Helicobacter pylori* antigen test results for gastric and oral *Helicobacter pylori* before treatment. HPS: *Helicobacter pylori* antigen test; UBT: Urea breath test.

Table 2 Results of *Helicobacter pylori* antigen test and <sup>13</sup>C-urea breath test testing *n* (%)

HPS	<sup>13</sup> C-UBT	
	Positive	Negative
Peptic ulcer ( <i>n</i> = 54)		
Positive	29 (53.7)	8 (14.8)
Negative	14 (25.9)	3 (5.6)
Chronic atrophic gastritis ( <i>n</i> = 48)		
Positive	24 (50.0)	14 (29.1)
Negative	8 (16.7)	2 (4.2)
Duodenitis ( <i>n</i> = 58)		
Positive	35 (60.3)	16 (27.6)
Negative	2 (3.4)	5 (8.7)
Superficial gastritis ( <i>n</i> = 231)		
Positive	79 (34.2)	89 (38.5)
Negative	25 (10.8)	38 (16.5)
Control group ( <i>n</i> = 40)		
Positive	12 (30.0)	21 (52.5)
Negative	5 (12.5)	2 (5.0)

HPS: *Helicobacter pylori* antigen test; <sup>13</sup>C-UBT: <sup>13</sup>C-urea breath test.

O+G+tm group *vs* O+G+tmp group, *P* = 0.924) (Table 3).

There were some subtle differences in the rate of gastric *H. pylori* eradication between the different gastric disease groups. The eradication rates in the peptic ulcer and chronic atrophic gastritis groups were higher than those in the duodenitis and superficial gastritis groups, but the differences were not statistically significant (Table 4).

### Prevalence of oral *H. pylori* after treatment

After treatment, the prevalence of oral *H. pylori* in the O+G+tm group was significantly higher than those in the other 3 groups: O-G+tm group *vs* O+G+tm group (*P* < 0.0001), O+G+tm group *vs* O+G+tm group (*P* = 0.011), and O+G+tmp group *vs* O+G+tm group (*P* = 0.006). There was no significant difference between the O+G+tm and O+G+tmp groups (*P* = 0.790) (Table 3).

## DISCUSSION

The idea that *H. pylori* can be transmitted by both oral

Table 3 Eradication rate of gastric *Helicobacter pylori* and prevalence of oral *Helicobacter pylori* after treatment in all groups *n* (%)

Group	Eradication rate of gastric <i>Helicobacter pylori</i>	Prevalence of oral <i>Helicobacter pylori</i>
O-G+tm	42 (93.3) <sup>a,c</sup>	0 (0.0) <sup>a</sup>
O+G+tm	40 (78.4)	39 (76.5)
O+G+tm	54 (90.0) <sup>b</sup>	32 (53.3) <sup>a</sup>
O+G+tmp	54 (94.7) <sup>a,b,c</sup>	29 (50.9) <sup>a</sup>

<sup>a</sup>*P* < 0.05 *vs* O+G+tm group; <sup>b</sup>*P* > 0.05 *vs* O-G+tm group; <sup>c</sup>*P* > 0.05 *vs* O+G+tm group.

to oral and stomach to oral routes has been recognized since 1989 when Krajce first isolated *H. pylori* from the dental plaque of patients with gastric diseases related to *H. pylori* infection. Further studies have shown that the gastric *H. pylori* eradication rate in patients with oral *H. pylori* infection is lower than that in patients without oral *H. pylori* infection. With the increase in antibiotic resistance which has occurred during the past 10 years, the rate of gastric *H. pylori* eradication following triple therapy has significantly decreased. From 1983-1997, the average eradication rate for gastric *H. pylori* was 75%-90%<sup>[17]</sup>, but later decreased to 68.8% in the period from 1996 to 2005<sup>[18]</sup>. Improvements in the gastric *H. pylori* eradication rate produced by killing oral *H. pylori* can reduce the application of antibiotics<sup>[9,14]</sup>, which not only reduces the economic burden of treatment, but also lowers the risk of increasing the resistance of *H. pylori* to antibiotics. Oral *H. pylori* is mainly present in periodontal pockets and dental plaque<sup>[19,20]</sup>. Zaric *et al*<sup>[9]</sup> collected dental plaque and gastric tissues and examined them for *H. pylori* using nested polymerase chain reaction (PCR). In our study, for patients with both oral and gastric *H. pylori*, periodontal treatment was applied in addition to triple therapy. Ultrasonic waves were used to remove dental plaque and calculi, and root surface scaling was used to eradicate the periodontal pockets. Glucose chlorhexidine solution (0.15%) was used to lavage the periodontal pocket. After periodontal treatment, the gastric *H. pylori* eradication rate was increased from 47.6% to 77.3%, while the prevalence of oral *H. pylori* was decreased from 66.7% to 27.0%; these results indicated that periodontal treatment could kill oral *H. pylori*. The methods used by Zaric were useful, but not easy to employ, and the monetary cost was high. Conducting nested PCR requires specific instruments, and the periodontal treatment also requires experienced dentists; therefore, these methods are not practical for routine clinical application. The current study was conducted to seek a cost-effective method for diagnosing oral *H. pylori* infection and reducing its prevalence.

Currently, there are two methods (bacterial culturing and nested PCR) used to diagnose oral *H. pylori* infection. Although the bacterial culture method has high specificity, large numbers of other oral bacteria can inhibit the growth of *H. pylori*, leading to a high false negative rate<sup>[21]</sup>. The nested PCR method has high sensitivity and

**Table 4** Eradication rate of gastric *Helicobacter pylori* after treatment in all gastric disease groups *n* (%)

Group	Gastric diseases				
	Peptic ulcer	Chronic atrophic gastritis	Duodenitis	Superficial gastritis	Control group
O+G+t	10 (100.0)	6 (100.0)	2 (100.0)	19 (86.4)	5 (100.0)
O+G+t	9 (90.0)	7 (87.5)	8 (72.7)	12 (70.6)	4 (80.0)
O+G+tm	9 (100.0)	10 (100.0)	9 (75.0)	22 (88.0)	4 (100.0)
O+G+tmp	9 (100.0)	5 (100.0)	10 (91.0)	27 (93.1)	3 (100.0)

specificity, but the results have been highly variable due to the use of different primers, and it cannot differentiate between DNA from dead and living bacteria. *H. pylori* DNA can still be detected even if the bacteria are already dead<sup>[22,23]</sup>. Thus, the nested PCR method cannot monitor the therapeutic efficacy of a treatment for oral *H. pylori*.

The HPS test is a rapid immunochromatographic assay. The principle of this test kit is based on using a colloidal gold chromatography double antibody sandwich to detect *H. pylori* flagellae and urease in human saliva. There are no cross-reactions with the urease released by other oral bacteria<sup>[14]</sup>.

Our results showed that the positive rate of HPS testing was 74.9%, demonstrating that the mouth is another storage site for *H. pylori*. In this study, the gastric *H. pylori* eradication rate in patients who were HPS+ was lower than that in patients who were HPS- (78.4% *vs* 93.3%). We also noted that the test results of gastric and oral *H. pylori* were not consistent. Previous studies have shown that *H. pylori* does not colonize in the mouth of a person who practices good oral hygiene (*e.g.*, no periodontal disease, no gingival band or plaque)<sup>[24]</sup>. In this situation, the oral *H. pylori* titer is low and does not reach the threshold of gastric *H. pylori* infection. Therefore, a test to detect gastric *H. pylori* infection would give negative results. For gastric *H. pylori*-positive patients with good oral hygiene, although gastric *H. pylori* may be refluxed into the mouth, the bacterium may not survive in the mouth. In this study, the HPS test had a high sensitivity, which enabled it to detect a low titer of *H. pylori*. Therefore, the positive rate for oral *H. pylori* infection was higher than that for gastric *H. pylori* infection.

*H. pylori* in the oral cavity is covered by a special protective shell called a biofilm, and systemic eradication therapy may not be very effective when used for treatment<sup>[25,26]</sup>; this study confirmed that hypothesis. The gastric *H. pylori* eradication rate in the O+G+t group was much higher than that in the oral *H. pylori* group (78.4% *vs* 23.5%). Compared with the O+G+t group, the eradication rates of gastric *H. pylori* in the O+G+tm and O+G+tmp groups were elevated from 78.4% to 90.0% and 94.7%, respectively, while the prevalence of oral *H. pylori* was reduced from 76.5% to 53.3% and 50.9%, respectively. There was no significant difference in gastric *H. pylori* eradication rate between the O+G+tm group and the O+G+tmp group. The gastric *H. pylori* eradication rates in the peptic ulcer group, chronic atrophic gastritis group, and control group were higher than those in the duodenitis group and superficial gastritis group, but no conclusion could be drawn from this finding because the

number of patients was small. This study demonstrated that mouthrinse treatment alone or combined with periodontal treatment could effectively reduce the prevalence of oral *H. pylori*, but did not prove a mechanism for this result. One hypothesis may be that the mouthrinse treatment or periodontal treatment reduced the amount of dental plaque and improved oral hygiene, thus enabling the titer of *H. pylori* to be reduced.

This study proved that mouthrinse treatment alone or combined with periodontal treatment could reduce the prevalence of oral *H. pylori* and improve the eradication rate of gastric *H. pylori*. The HPS test is a simple and rapid method that can diagnose oral *H. pylori* infection; however, the test results cannot be analyzed quantitatively. Therefore, the therapeutic effect of various treatments could not be thoroughly analyzed.

## COMMENTS

### Background

It is well acknowledged that the stomach can serve as a reservoir for *Helicobacter pylori* (*H. pylori*) and that gastric *H. pylori* can be killed by triple or quadruple therapy. Increasing numbers of studies have suggested that the average eradication rate of gastric *H. pylori* has dropped in recent years, and it is important to find a practical way to improve the eradication rate.

### Research frontiers

Since Krajce isolated *H. pylori* from dental plaque in 1989, many studies have demonstrated that the oral cavity serves as an extra-gastric reservoir for *H. pylori*. Several studies have suggested that patients who test positive for oral *H. pylori* have a lower success rate of gastric *H. pylori* eradication than oral *H. pylori*-negative individuals. Zaric used nested polymerase chain reaction (PCR) to test for the presence of oral *H. pylori*, and improved the eradication rate of gastric *H. pylori* from 47.6% to 77.3% by removing dental plaque. However, the cost of this procedure was high and the process was complicated. There is a great need to find a simple and rapid diagnostic test that can be used to confirm the presence of oral *H. pylori* infection, and to identify a method that can increase the eradication rate of gastric *H. pylori* and is feasible for clinical application.

### Innovations and breakthroughs

<sup>13</sup>C-UBT is the gold standard for diagnosis of gastric *H. pylori* infection; however, a unified method has not been identified for diagnosing oral *H. pylori* infection. Nested PCR, which tests the DNA of *H. pylori*, is commonly used, but has a high false positive rate because it cannot distinguish between DNA from dead and living bacteria. The current study replaced the nested PCR method with a saliva *H. pylori* antigen test (HPS) to test for oral *H. pylori*. The HPS test employs a monoclonal antibody that was developed against semipurified *H. pylori* protein, and in another experiment, this test showed no interference or cross reactivity with other oral bacteria. Zaric killed oral *H. pylori* by removing dental plaque and lavaging the periodontal pocket, which required a professional dentist. In the current study, mouthwash was used to effectively kill oral *H. pylori*.

### Applications

This study used the saliva *H. pylori* antigen test to diagnose oral *H. pylori* infection. This test is simple to use and results are rapidly obtained. The eradication rate of gastric *H. pylori* improved significantly in patients who received mouth-

rinse treatment alone or combined with periodontal treatment. This innovative approach can not only reduce the economic burden of patients, but also decrease the development of resistance to antibiotics.

### Terminology

*H. pylori*: *H. pylori* is a spiral rod-shaped bacterium that mainly colonizes the epithelium of the stomach. It is considered to be the main cause of peptic ulcer, chronic active gastritis, gastroduodenal ulcer, mucosa-associated lymphoid tissue lymphoma and gastric cancer. Approximately half of the world's population is infected. Triple therapy which consists of one proton inhibitor and two antibiotics is often used to kill *H. pylori*.

### Peer review

This is an interesting study, indicating a cheap way of improving *H. pylori* eradication rate. The study was performed on 391 persons who underwent gastroscopy and histopathological examination of gastric mucosa. For evaluation of *H. pylori* in oral cavity, the authors used *H. pylori* saliva test based on detection of *H. pylori* antigen in saliva using rapid immunochromatographic assay. For evaluation of *H. pylori* in stomach mucosa, the authors used <sup>13</sup>C-urea breath test. The results showed that the eradication of *H. pylori* in the mouth cavity using mouth rinse and periodental treatment could kill the oral *H. pylori* and improve the eradication rate of gastric *H. pylori* by triple therapy. The study is set up correctly. The paper is written well. The Introduction gives a good overview of the study background and the authors raised clearly the aim of the study. The material studied is large enough to draw the conclusions. The Tables and Figure give a good overview about the results.

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## Importance of *b* value in diffusion weighted imaging for the diagnosis of pancreatic cancer

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### Abstract

**AIM:** To investigate the use of multi-*b*-value diffusion-weighted imaging in diagnosing pancreatic cancer.

**METHODS:** We retrospectively analyzed 33 cases of pancreatic cancer and 12 cases of benign pancreatic tumors at the Second Affiliated Hospital of Kunming Medical University from December 2008 to January 2011. The demographic characteristics, clinical presentation, routine magnetic resonance imaging and diffusion weighted imaging (DWI) features with different *b* values were reviewed. Continuous data were expressed as mean  $\pm$  SD. Comparisons between pancreatic cancer and benign pancreatic tumors were performed using the Student's *t* test. A probability of  $P < 0.05$  was considered statistically significant.

**RESULTS:** Thirty-three patients with pancreatic cancer were identified. The mean age at diagnosis was  $60 \pm 5.6$  years. The male: female ratio was 21:12. Twenty

cases were confirmed by surgical resection and 13 by biopsy of metastases. T1 weighted images demonstrated a pancreatic head mass in 16 patients, a pancreatic body mass in 10 cases, and a pancreatic tail mass with pancreatic atrophy in 7 cases. Eight patients had hepatic metastases, 13 had invasion or envelopment of mesenteric vessels, 4 had bone metastases, and 8 had lymph node metastases. DWI demonstrated an irregular intense mass with unclear margins. Necrotic tissue demonstrated an uneven low signal. A *b* of 1100 s/mm<sup>2</sup> was associated with a high intensity signal with poor anatomical delineation. A *b* of 700 s/mm<sup>2</sup> was associated with apparent diffusion coefficients (ADCs) that were useful in distinguishing benign and malignant pancreatic tumors ( $P < 0.05$ ). *b* values of 50, 350, 400, 450 and 1100 s/mm<sup>2</sup> were associated with ADCs that did not differentiate the two tumors.

**CONCLUSION:** Low *b* value images demonstrated superior anatomical details when compared to high *b* value images. Tumor tissue definition was high and contrast with the surrounding tissues was good. DWI was useful in diagnosing pancreatic cancer.

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**Key words:** Pancreatic cancer; Magnetic resonance imaging; *b* value; Apparent diffusion coefficient; Diffusion weighted imaging

**Core tip:** In this study, we retrospectively analyzed the conventional magnetic resonance imaging and diffusion weighted imaging (DWI) characteristics of 33 cases of pancreatic cancer using different *b* values, and assessed the value of the DWI examination in differentiating pancreatic cancer from benign pancreatic tumors.

Hao JG, Wang JP, Gu YL, Lu ML. Importance of *b* value in diffusion weighted imaging for the diagnosis of pancreatic cancer. *World J Gastroenterol* 2013; 19(39): 6651-6655 Available from:

## INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths<sup>[1]</sup> and accounts for 80% to 90% of exocrine gland malignant tumors<sup>[2]</sup>. Most patients present without symptoms and have a median survival of approximately 6 months. There is an urgent need for early diagnosis and accurate assessment of this disease. Magnetic resonance imaging (MRI) is a sensitive and specific imaging modality. MRI has been used to assess tumor macroscopic morphology, microscopic metabolism, and functional status<sup>[3,4]</sup>. Diffusion weighted imaging (DWI) is an imaging technique that is sensitive to water diffusion in living tissues. DWI was originally used to diagnose acute stroke<sup>[5,6]</sup>. DWI has also been used to diagnose liver, kidney, breast, prostate and uterine disease. DWI is frequently used to diagnose pancreatic diseases<sup>[7-10]</sup>. We retrospectively analyzed the conventional MRI and DWI characteristics of 33 patients with pancreatic cancer and 12 with benign pancreatic tumors to evaluate the value of DWI.

## MATERIALS AND METHODS

### Study patients

Thirty-three patients with pancreatic cancer were hospitalized at the Second Affiliated Hospital of Kunming Medical University between December 2008 and January 2011. Twenty patients had their diagnosis confirmed by pathological examination of the resected specimen and 13 by biopsy of metastases. There were 21 male and 12 female patients with an average age of  $60 \pm 5.6$  years. Sixteen patients had a mass in the pancreatic head, 10 in the pancreatic body, and 7 in the pancreatic tail. Clinical symptoms included abdominal pain, abdominal discomfort, jaundice, abdominal mass, significant weight loss and loss of appetite. Control cases with benign pancreatic tumors were confirmed by histopathology.

### Imaging data

Imaging was performed using a Siemens Sonata 1.5 T superconducting scanner with a body phased-array surface coil. A T1WI-FLASH sequence (repetition time, TR 124 ms and echo time, TE 2.47 ms) and T2WI-HASTE sequence (TR 1000 ms and TE 93 ms) were used with 18-24 layers, a thickness of 4-8 mm, spacing between 0 and 1.6 mm, and a field of view (FOV) of 240-280 mm  $\times$  300-380 mm. The matrix was 320  $\times$  256. Scan time was 13-18 s.

DWI scanning was performed using a SE-EPI sequence (TR 4000 ms, TE 85-95 ms, Matrix 128  $\times$  128, FOV 230 mm  $\times$  230 mm, thickness 5 mm, spacing 0.5 mm) with fat suppression, flow compensation and chemical shift saturation. The *b* value (apparent diffusion coefficient) was varied (50, 350, 400, 450, 700 and 1100 s/mm<sup>2</sup>) to capture images. Slice selection was performed

using frequency encoding and phase encoding in 3 directions. Images were processed using MR software. Scan time was 13-18 s.

### Data analysis

The original DWI scanning data and automatically generated apparent diffusion coefficients (ADC) were transferred to the workstation. The value of the ADC was measured from the ADC image of each region of interest (ROI). Solid tumor ROIs were not less than half of the lesion and located in the center of the mass. Areas of necrosis, the main pancreatic duct, vascular branches and chemical shift artifacts were avoided. Three ADCs were measured from each ROI and averaged.

### Statistical analysis

All statistical analyses were performed using SPSS, version 17.0 for Windows. Continuous data were expressed as mean  $\pm$  SD. The differences in ADC values of pancreatic cancer and benign pancreatic tumors were evaluated using the Student's *t* test. All reported *P* values were two-sided. *P* < 0.05 was considered statistically significant.

## RESULTS

### Conventional MRI-T1WI

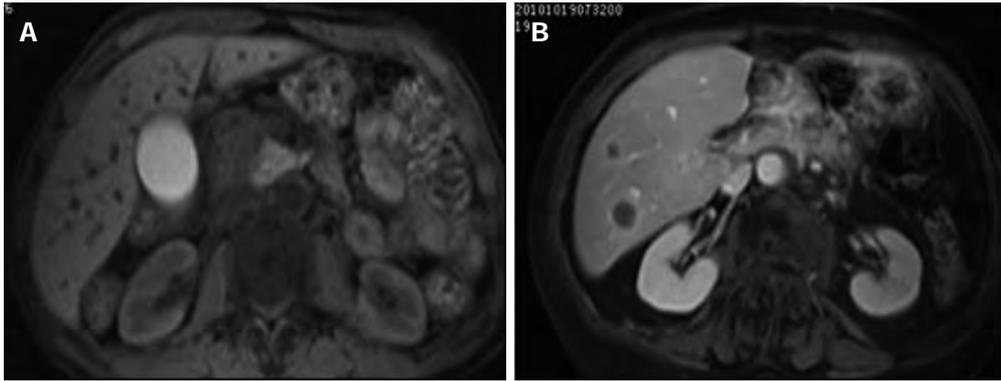
Sixteen patients had a pancreatic head mass, with a local or diffuse low intensity signal (Figure 1A). 10 had a pancreatic body mass with a low intensity signal (Figure 1B) and 7 patients had a pancreatic tail mass with pancreatic atrophy. Eight patients had liver metastases, 13 demonstrated invasion into or enveloping local mesenteric vessels, 4 had bone metastases and 8 had lymph node metastases.

### DWI

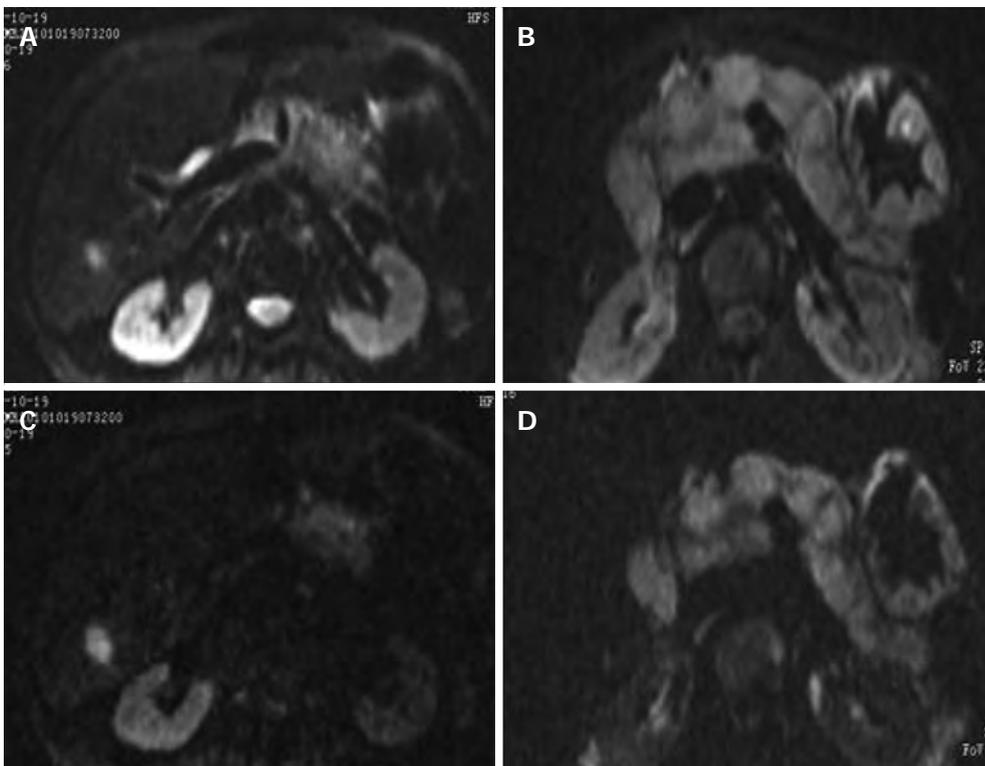
DWI demonstrated an uneven intense signal with margins that were not clearly delineated. The central necrotic tissue had an irregular low intensity signal. A low *b* value image provided better anatomical detail than a high *b* value image. Tumor tissue definition was high, and there was sharp contrast with the surrounding tissue (Figure 2). A *b* of 1100 s/mm<sup>2</sup> was associated with a high value signal and poor definition of anatomic structures. A *b* of 700 s/mm<sup>2</sup> was associated with benign pancreatic tumors and pancreatic cancer ADCs that were significantly different (*P* < 0.05). The two tumor types had similar ADCs when *b* values of 50, 350, 400, 450 and 1100 s/mm<sup>2</sup> were used for imaging (*P* > 0.05) (Table 1).

## DISCUSSION

Pancreatic cancer is one of the most common malignant tumors of the pancreas, accounting for approximately 75%-90% of tumors. It is the most common gastrointestinal malignant tumor<sup>[10]</sup>. The retroperitoneal location and lack of symptoms prevents early detection. Pancreatic cancers have a poor prognosis, with a five year survival of only 1%-3%<sup>[11]</sup>. Patients are generally male and 40-70



**Figure 1** T1 weighted image. A: T1 weighted image. The margins were not sharp; B: T1 weighted image with contrast. There was obvious enhancement. Two nodules in the right liver demonstrated ring enhancement.



**Figure 2** Tumor tissue definition was high, and there was sharp contrast with the surrounding tissue. A:  $b = 50 \text{ s/mm}^2$ ; B:  $b = 400 \text{ s/mm}^2$ ; C:  $b = 700 \text{ s/mm}^2$ ; D:  $b = 1100 \text{ s/mm}^2$ . A high intensity signal was seen.

**Table 1** Evaluation of apparent diffusion coefficients in pancreatic cancer and benign pancreatic tumors using different  $b$  values

$b \text{ (s/mm}^2\text{)}$	Apparent diffusion coefficients ( $10^{-3} \text{ s/mm}^2$ )	
	Benign pancreatic tumors	Pancreatic cancer
50	$2.273 \pm 0.298$	$2.006 \pm 0.194$
350	$1.705 \pm 0.227$	$1.489 \pm 0.306$
400	$1.590 \pm 0.553$	$1.376 \pm 0.276$
450	$1.544 \pm 0.194$	$1.333 \pm 0.218$
700	$1.519 \pm 0.125^1$	$1.118 \pm 0.102^1$
1100	$1.380 \pm 0.249$	$1.085 \pm 0.163$

<sup>1</sup>Indicates statistically significant difference,  $P < 0.05$ .

years of age. Only a minority of patients are candidates for surgery at the time of diagnosis<sup>[12]</sup>.

The majority of pancreatic cancers are adenocarcinomas. Ductal adenocarcinomas account for 85%-90% of pancreatic carcinomas and originate in the ductal epithelium. Ductal adenocarcinomas are avascular solid tumors that are locally invasive. About 70% of pancreatic cancers are located in the pancreatic head, neck and uncinate process, 20% are located in the body of the pancreas, and 5%-10% are located in the tail of the pancreas.

Abdominal imaging is used to diagnose pancreatic tumors, distinguish benign and malignant pancreatic tumors, and evaluate the resectability of pancreatic cancers

before surgery<sup>[13-15]</sup>. Endoscopic ultrasound (EUS) with zone sonography technology has been used in the diagnosis of pancreatic disease<sup>[16]</sup>. The sensitivity of EUS fine needle aspiration for pancreatic adenocarcinoma<sup>[17]</sup> in early studies was more than 85%. Further studies are needed. Egorov *et al*<sup>[18]</sup> demonstrated the utility of combined CT and EUS in the detection of arterial involvement by pancreatic cancer. Previous studies<sup>[19]</sup> have shown that DWI performed significantly better than multidetector-row CT in the detection of liver metastases in patients with pancreatic tumors. PET has also been useful as a diagnostic and predictive tool, but its efficacy in the staging of pancreatic cancer is not known<sup>[20]</sup>. A meta-analysis of pancreatic imaging<sup>[21]</sup> suggested that DWI was a potentially useful modality for differentiating malignant from benign pancreatic lesions. There are few studies on the effect of *b* value on DWI in the diagnosis of pancreatic cancer. Normal pancreatic tissue contains more water than pancreatic cancer, resulting in a high T1 weighted signal. Tumor liquefaction, necrosis and hemorrhage are associated with an irregular low intensity signal. T2 weighted images are mainly used to evaluate fluid composition, pancreatic duct dilation, and pseudocyst formation. These images are not specific for pancreatic cancer, eliciting low and high intensity signal. DWI is a noninvasive magnetic resonance imaging method, which can detect the irregular random movement of water molecules<sup>[22]</sup>. DWI can provide spatial information and evaluate the exchange rate of water molecules in tissues. ADCs have been used to describe and measure the activity of water molecules.

*b* values of 50 and 350 s/mm<sup>2</sup> were associated with clear DWIs, but the ADC value was not precise. With small *b* values, the proportion of diffusion is small and blood perfusion had a greater impact on DWI. While T2 was associated with an intense signal, DWI did not show a good margin between tumors and the surrounding tissue<sup>[23-25]</sup>. These factors affect the quality of DWI and the measurement of ADC.

DWI and ADCs with small *b* values were not useful in diagnosing pancreatic tumors. A *b* value of 1100 s/mm<sup>2</sup> was not useful in generating ADCs that could differentiate benign and malignant pancreatic tumors. This may be due to a decline in image quality seen with high *b* values. A *b* value of 700 s/mm<sup>2</sup> was useful in generating ADCs that could differentiate the two tumor types. The amount of tumor fibrosis, necrosis, cell proliferation, and changes in the nuclear/cytoplasmic ratio and membranous structure restricted the movement of water molecules in pancreatic cancers, decreasing the ADC values<sup>[26]</sup>.

The small sample size of this study increases the possibility of a type 2 error. Randomized controlled trials are needed to verify the utility of specific *b* values to aid in the differential diagnosis of pancreatic cancer.

In conclusion, low *b* value imaging demonstrated anatomical details that were superior to high *b* value images. Tumor tissue definition was high and contrast with the surrounding tissue was good. DWI was useful in diagnos-

ing pancreatic cancer.

## COMMENTS

### Background

Pancreatic cancer is the fourth leading cause of cancer-related deaths. Early detection, early diagnosis, and suitable treatment play an important role in extending patient survival. Diffusion weighted imaging (DWI) is a technically feasible measure to differentiate malignant from benign pancreatic lesions.

### Research frontiers

DWI is a magnetic resonance imaging technique that can be used to evaluate liver, kidney, breast, prostate and uterine tissue, and is especially useful in evaluating the upper abdomen.

### Innovations and breakthroughs

The authors retrospectively analyzed the DWI characteristics of 33 cases of pancreatic cancer using multiple *b* values in order to identify the optimal *b* value for differentiating malignant from benign pancreatic lesions.

### Applications

The authors found that low *b* values provided superior anatomical details, a quality image, good tumor tissue definition, and good contrast with the surrounding tissue.

### Terminology

DWI is an imaging technique sensitive to water molecule diffusion. It can non-invasively evaluate diffusion processes inside living cells.

### Peer review

This is an interesting study with great promise. DWI appears useful in diagnosing pancreatic cancer.

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## Macrophage migration inhibitory factor gene polymorphisms in inflammatory bowel disease: An association study in New Zealand Caucasians and meta-analysis

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### Abstract

**AIM:** To investigate the association of macrophage migration inhibitory factor (MIF) promoter polymorphisms with inflammatory bowel disease (IBD) risk.

**METHODS:** One thousand and six New Zealand Caucasian cases and 540 Caucasian controls were genotyped for the *MIF* SNP -173G > C (rs755622) and the repeat polymorphism CATT<sub>5-8</sub> (rs5844572) using a pre-designed TaqMan SNP assay and capillary electrophoresis, respectively. Data were analysed for single site and haplotype association with IBD risk and phenotype. Meta-analysis was employed, to assess cumulative evidence of association of *MIF* -173G > C with IBD. All published genotype data for *MIF* -173G > C in IBD were identified using PubMed and subsequently searching the references of all PubMed-identified studies. Imputed genotypes for *MIF* -173G > C were generated from the Wellcome Trust Case Control Consortium (and National Institute of Diabetes and Digestive and Kidney Diseases). Separate meta-analyses were performed on Caucasian Crohn's disease (CD) (3863 patients, 6031 controls), Caucasian ulcerative colitis (UC) (1260 patients, 1987 controls), and East Asian UC (416 patients and 789 controls) datasets using the Mantel-Haenszel method. The New Zealand dataset had 93% power, and the meta-analyses had 100% power to detect an effect size of OR = 1.40 at  $\alpha = 0.05$ , respectively.

**RESULTS:** In our New Zealand dataset, single-site analysis found no evidence of association of MIF polymorphisms with overall risk of CD, UC, and IBD or disease phenotype (all *P* values > 0.05). Haplotype analysis found the CATT<sub>5</sub>/-173C haplotype occurred at a higher frequency in New Zealand controls compared to IBD patients (0.6 vs 0.01; *P* = 0.03, OR = 0.22; 95%CI: 0.05-0.99), but this association did not survive bonferroni correction. Meta-analysis of our New Zealand *MIF* -173G > C data with data from seven additional Caucasian datasets using a random effects model found no association of *MIF* polymorphisms with CD, UC, or overall IBD. Similarly, meta-analysis of all pub-

lished *MIF* -173G > C data from East Asian datasets (416 UC patients, 789 controls) found no association of this promoter polymorphism with UC.

**CONCLUSION:** We found no evidence of association of MIF promoter polymorphisms with IBD.

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**Key words:** Crohn's disease; Ulcerative colitis; Migration inhibitory factor; rs755622; rs5844572; Genetic association study

**Core tip:** Migration inhibitory factor (MIF) is an important mediator of inflammatory bowel disease (IBD). However, whether promoter polymorphisms in MIF alter susceptibility to IBD is unclear. This study sought to clarify this, as definitively as possible, for Caucasians and East Asians. Analysis of a New Zealand Caucasian cohort found no association of the polymorphisms *MIF* -173G > C and *CATT*<sub>5-8</sub> with IBD. Subsequent meta-analysis of the New Zealand data with published MIF -173G > C data from other Caucasian cohorts found no association. A separate meta-analysis of East Asian datasets also found no evidence of association of this promoter polymorphism with IBD.

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## INTRODUCTION

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine implicated in the pathophysiology of numerous inflammatory conditions including inflammatory bowel disease (IBD)<sup>[1]</sup>. MIF is a widely expressed component of the immune system that is released in response to diverse stimuli including lipopolysaccharide (*via* toll-like receptor-4, TLR-4), pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  and interferon-gamma, and hypoxia<sup>[2,3]</sup>. MIF in turn up-regulates the expression of TLR-4, pro-inflammatory cytokines, and acts as a co-factor for the activation of T-cells<sup>[4]</sup>. MIF is elevated in the plasma of patients with active IBD and falls following successful treatment<sup>[5,6]</sup>. In murine models, transgenic over-expression of MIF increases susceptibility to IBD while *MIF* knockout mice are protected from the development of colitis<sup>[5-8]</sup>. Moreover, the ability of neutralising anti MIF antibodies to ameliorate murine colitis indicates the potential value of MIF as a therapeutic target<sup>[5,6,8]</sup>.

A single nucleotide polymorphism (SNP) -173G > C (rs755622) and a tetra-nucleotide repeat *CATT*<sub>5-8</sub>

(rs5844572) have been identified in the *MIF* promoter<sup>[4,9]</sup>. These polymorphisms are associated with increased plasma concentrations of MIF, increased risk and severity of inflammatory disease, and reduced response to glucocorticoid medication<sup>[4,9,10]</sup>. The functional effect of *MIF*-173G > C is attributed to the creation of a binding site for the transcription factor AP4<sup>[11]</sup>, whilst the mechanism by which *CATT*<sub>5-8</sub> alters promoter activity is unknown. Despite several previous studies<sup>[12-19]</sup>, the genetic contribution of *MIF* promoter polymorphisms to IBD susceptibility and phenotype is unclear. The overall aim of this study was to clarify, as definitively as possible, the contribution of *MIF* promoter polymorphisms to IBD risk and phenotype in Caucasians and East Asians. To achieve this aim, we conducted the largest single-dataset study of *MIF* promoter polymorphisms -173G > C and *CATT*<sub>5-8</sub> in Caucasians to date, and meta-analysed these new data with previously published data to test for cumulative evidence of association with IBD risk and phenotype in Caucasians and East Asians.

## MATERIALS AND METHODS

### Ethics

All New Zealand study participants gave their informed written consent and approval for the study was obtained from the Upper and Lower South Regional Ethics Committees of New Zealand (Approval ID: CTY/03/01/011; Date: 05/06/2007). For the meta-analysis, genotype data from non-New Zealand subjects were obtained from review of published studies (Table 1).

### New Zealand controls and IBD patients

Study participants were selected from a population-based study of genetic and environmental determinants of the aetiology of IBD in the Canterbury region of New Zealand which has been described in detail elsewhere<sup>[20]</sup>. The population of Canterbury is largely of Northern European (United Kingdom and Irish) origin and participants were included in the current study if they were of self-reported European ancestry. Diagnosis of IBD was made by standard criteria<sup>[21]</sup>. Detailed phenotypic data according to the Montreal Classification system were available in addition to information regarding the presence of extra-intestinal manifestations of disease, history of immunomodulator use and the need for surgery<sup>[21]</sup>. The control group comprised 540 healthy Caucasian New Zealanders over the age of 17 years with no history of inflammatory disorders<sup>[22]</sup>.

### MIF genotyping

DNA was collected from peripheral blood samples of the IBD patients and controls using guanidinium isothiocyanate-chloroform extraction<sup>[23]</sup>. Genotyping of *MIF* -173G > C (rs755622) was performed using a pre-designed Taqman<sup>®</sup> SNP genotyping assay (assay ID: C\_2213785\_10) as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, United States).

**Table 1 Characteristics of previously published association studies of MIF -173G > C in inflammatory bowel disease**

Cohort	Sample size	Demographics			Ref.	
		Race	mean ± SD	% male		IBD diagnosis
1	Cases: 111	Japanese	39 ± 14.2	52.3	UC	[18]
	Controls: 209		44 ± 18.4	58.4	NA	
2	Cases: 221	Japanese	40.1 ± 14.0	46.2	UC	[15]
	Controls: 312		41.4 ± 18.1	50.3	NA	
3	Cases: 99	Han Chinese	38.4 ± 10.6	61.6	CD (15); UC (84)	[13]
	Controls: 142		42.3 ± 11.6	64.1	NA	
4	Cases: 623	Caucasian	-	48 (CD); 49.0 (UC)	CD (336); UC (287)	[16]
	Controls: 361		-	-	NA	
5	Cases: 672	Caucasian	-	47.1 (CD); 43.0 (UC)	CD (325); UC (347)	[16]
	Controls: 526		-	-	NA	
6	Cases: 99	Caucasian	-	-	41 (CD); 58 (UC)	[17]
	Controls: 123		-	-	NA	
7	Cases: 198	Caucasian	39.5 ± 11.9	48.0	CD	[12]
	Controls: 159		44.1 ± 15.8	46.5	NA	
8	Cases: 259	Caucasian	42.3 ± 10.2 (CD); 44.1 ± 15.3 (UC)	-	157 (CD), 102 (UC)	[14]
	Controls: 489		-	-	NA	
9	Cases: 1234	Caucasian	-	47.2	CD	[32]
	Controls: 2112		-	51.0	NA	
10	Cases: 590	Caucasian	21 ± 10.8	47.2	CD	[33,34]
	Controls: 678		-	49.3	NA	
12	Cases: 488	Caucasian (Indian)	44.9 ± 11.2	41.2	NA	[19] <sup>1</sup>
	Controls: 176		41.0 ± 14.11	59.3	139 (UC) NA	

<sup>1</sup>The study of Sivaram *et al*<sup>[19]</sup> was not included in the meta-analyses as study participants were neither East Asian, nor Northern European or American Caucasians. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; NA: Not available.

The *MIF* CATT<sub>5-8</sub> repeat, was amplified in a total volume of 10 µL containing 2 mM dNTPs (Fisher Biotech), 2 mM MgCl<sub>2</sub>, 10% betaine (Sigma, St Louis, Missouri, United States), 0.5 µmol/L of the primers MIFrepf (5' FAM-gcctgtgatccagttgctgctgtgc3') and MIFrepr (5'ccac-taatgtaaaactcggggaccat3'), 1 U of Platinum Taq DNA Polymerase (Invitrogen, CA, United States), and 20 ng of genomic DNA. The forward primer, MIFrepf, was labelled to enable subsequent resolution on a DNA Analyzer (Applied Biosystems 3730xl DNA Analyzer). The polymerase chain reaction (PCR) conditions comprised an initial denaturation step of 2 min at 94 °C; followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s; and a final extension step of 1 min at 72 °C. PCRs were diluted by the addition of 20 µL of water. Amplification of the *MIF* promoter was assessed by 3% agarose gel electrophoresis. To accurately size the PCR products and thus reliably assign CATT genotypes, 2 µL of each diluted PCR product was mixed with 1 µL of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems, Foster City, CA, United States) and resolved on an ABI 3730xl DNA Analyzer. Samples containing 5, 6, 7, or 8 CATT repeats yielded PCR products of 152, 156, 160 and 164 bp, respectively. Genotyping results were then analysed using Peak Scanner™ Software version 1 (Applied Biosystems, Foster City, CA, United States). Accuracy of the TaqMan SNP and CATT assays was confirmed by repeat analysis of 10% of samples. The concordance between original and repeat genotypes was 100%.

### Association testing

The software package PLINK<sup>[24]</sup> was used to test for deviations from Hardy-Weinberg Equilibrium (HWE), and to conduct single marker and haplotype association tests. The statistical significance of the observed allele and genotype associations were determined by Pearson's  $\chi^2$ . Results were considered statistically significant if *P* was < 0.05. Bonferroni's correction was applied to adjust for multiple testing (0.05/18 tests, adjusted *P* < 0.0028). As CATT<sub>5</sub> is reported to have less promoter activity than longer repeat alleles<sup>[9,25]</sup>, tests of association were performed on full genotype and following simplification to 55, 5X or XX, where X = CATT<sub>6-8</sub>, as previously described<sup>[9,26,27]</sup>.

### Imputation and meta-analysis

All published studies of *MIF* -173G > C and *MIF* CATT<sub>5-8</sub> in IBD were identified using PubMed and subsequently searching the references of all PubMed-identified studies. Imputed genotypes for *MIF* -173G > C were generated from the Wellcome Trust Case Control Consortium (WTCCC)<sup>[28]</sup> and National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Crohn's disease (CD) datasets<sup>[29,30]</sup> using IMPUTE and the HapMap release [NCBI dbSNP Build 131 (Apr 2010, hg37.1)]. A quality threshold of 0.95 was set for imputation of both datasets.

Meta-analyses were conducted for the *MIF* SNP -173G > C in STATA (Stata Statistics/Data Analysis Software, version 8, Stata Corporation, Texas, United

**Table 2** Baseline characteristics of the New Zealand inflammatory bowel disease patients and controls *n* (%)

Characteristic	Controls	CD	UC	IBD-U
Number of females	340 (63.0)	307 (63.0)	242 (51.5)	13 (50.0)
Age at first diagnosis (yr)				
< 17		57 (11.3)	28 (5.9)	
17-40		277 (55.0)	231 (48.6)	15 (55)
> 40		170 (33.7)	216 (45.5)	12 (45)
CD location				
Colonic		211 (41.8)		
Ileocaecal		125 (24.8)		
Ileal		64 (32.5)		
Isolated upper GI disease		3 (0.6)		
Perianal disease modifier		136 (27.0)		
CD behaviour				
Non-stricturing/non-penetrating		286 (56.8)		
Penetrating		57 (11.3)		
Stricturing		161 (31.9)		
UC location				
Proctitis			164 (34.5)	3 (11.1)
Left-sided			125 (26.3)	5 (18.5)
Extensive			181 (38.1)	19 (70.4)

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn’s disease; GI: Gastrointestinal.

States) using the Mantel-Haenszel (MH) method with either a random or a fixed effects model depending on the presence or absence respectively of significant heterogeneity between studies ( $P < 0.05$ ). Meta-analyses were considered significant if the pool MH  $P$  value was  $< 0.05$ . As published CATT<sub>5-8</sub> frequency data was limited to a single Japanese ulcerative colitis (UC) dataset<sup>[18]</sup>, meta-analysis was not conducted for this variant.

## RESULTS

### Single-site analysis of MIF polymorphisms

MIF-173G > C genotype was determined in 988 New Zealand patients (495 CD, 466 UC and 27 IBD-U) and 488 New Zealand controls, and CATT<sub>5-8</sub> genotype was determined in 975 New Zealand patients (476 CD, 473 UC and 26 IBD-U) and 535 New Zealand controls. The baseline characteristics of the cases are shown in Table 2. Genotype and allele frequencies are shown in Table 3 (MIF-173G > C) and Table 4 (MIF CATT<sub>5-8</sub>). No significant deviation from HWE was observed in the patients or controls. Single-site analysis found no evidence of association of MIF-173G > C or CATT<sub>5-8</sub>, and CD, UC or overall risk of IBD (Tables 3 and 4). Furthermore, neither polymorphism was significantly associated with age at first diagnosis, disease location, or disease behaviour (penetrating, stricturing or non-penetrating/non-stricturing) in our New Zealand dataset.

### Haplotype analysis

Nine hundred and fifty-seven New Zealand patients (CD 467, UC 464, IBD-U 26) and 483 New Zealand

controls were successfully genotyped for both MIF promoter polymorphisms. Haplotype analysis of these polymorphisms found the MIF CATT<sub>5</sub>/-173C haplotype occurred at a higher frequency in controls compared to IBD patients (0.6 *vs* 0.01;  $P = 0.03$ , OR = 0.22, 95%CI: 0.05-0.99). However, after bonferroni correction for multiple testing (0.05/18 tests) no association of haplotype with susceptibility to CD, UC or IBD, or with disease phenotype, behaviour or clinical course was found.

### Meta-analyses of MIF-173G > C in Caucasian and East Asian IBD datasets

Meta-analysis was undertaken in order to determine the overall influence of MIF -173G > C on IBD susceptibility in Caucasians and East Asians. Published summary genotype data were available for five Caucasian<sup>[12,14,16,17]</sup>, and three East Asian datasets<sup>[13,15,18]</sup>. In addition, MIF-173G > C genotypes were imputed from both the WTCCC and NIDDK Caucasian CD datasets. As the contribution that specific loci make to IBD susceptibility differs significantly with race, the East Asian and Caucasian datasets were meta-analysed separately. The Breslow-Day test revealed the existence of significant heterogeneity ( $P_{het} \leq 0.05$ ) among the Caucasian datasets but not among the East Asian datasets ( $P_{het} = 0.5$ ). As a result a random effects model was applied to the Caucasian meta-analysis whilst a fixed effects model was applied to the East Asian meta-analysis. No cumulative evidence of association of MIF -173G > C with UC, CD or all IBD risk was detected in either Caucasians or East Asians (Figure 1).

## DISCUSSION

### Functional effects of MIF polymorphisms

MIF is a pleiotropic pro-inflammatory cytokine implicated in the pathophysiology of numerous diseases including IBD<sup>[5,9,31-33]</sup>. Promoter polymorphisms in MIF influence both basal and disease-associated MIF expression, and MIF expression has been shown to have profound effects on disease phenotype in experimental models<sup>[5,7,34]</sup>. *In vitro* MIF -173C allele exhibits greater expression than the MIF -173G allele in T lymphoblast cells, and expression of MIF increases with increasing length of the CATT repeat in COS-7 fibroblast like cells<sup>[9]</sup>. Despite these effects on expression, previous association studies investigating these polymorphisms in IBD have given discordant results<sup>[12-19]</sup>.

### Previously reported associations of MIF-173G > C and CATT<sub>5-8</sub> polymorphisms with UC and CD

The MIF-173C allele has been significantly associated with increased UC susceptibility in Spanish and Polish datasets<sup>[16,17]</sup>, but not in German<sup>[14]</sup> or Indian<sup>[19]</sup> datasets. The MIF-173 C/C genotype was significantly associated with pan-colitis compared with left-sided or distal disease (OR = 10.78, 95%CI: 1.34-86.62.  $P = 0.0074$ ) in one Japanese UC dataset ( $n = 221$ )<sup>[15]</sup>, but not a second Japanese

**Table 3 Allele and genotype frequencies of MIF-173G > C in New Zealand Caucasian patients with inflammatory bowel disease compared with controls**

Phenotype	Genotype			MAF	Allelic <i>P</i> (unadjusted)	OR (95%CI)
	GG	GC	CC			
Control	328 (0.672)	143 (0.293)	17 (0.035)	177 (0.181)		
IBD	668 (0.676)	287 (0.290)	33 (0.033)	353 (0.179)	0.86	0.98 (0.80-1.20)
UC	320 (0.687)	129 (0.277)	17 (0.036)	163 (0.175)	0.71	0.96 (0.76-1.21)
Age at first diagnosis (yr)						
< 17	17 (0.610)	11 (0.390)	0	11 (0.196)	0.78	1.10 (0.56-2.18)
17-40	153 (0.680)	60 (0.267)	12 (0.053)	84 (0.187)	0.81	1.04 (0.78-1.38)
> 40	150 (0.704)	58 (0.272)	5 (0.023)	68 (0.160)	0.33	0.86 (0.63-1.17)
Disease location						
Proctitis	106 (0.658)	49 (0.304)	6 (0.037)	61 (0.189)	0.75	1.06 (0.76-1.46)
Left-sided	92 (0.742)	27 (0.218)	5 (0.040)	37 (0.149)	0.23	0.79 (0.54-1.16)
Extensive	118 (0.670)	52 (0.295)	6 (0.034)	64 (0.182)	0.99	1.00 (0.73-1.38)
CD	331 (0.669)	149 (0.301)	15 (0.030)	179 (0.181)	0.98	0.00 (0.79-1.25)
Age at first diagnosis (yr)						
< 17	36 (0.642)	19 (0.339)	1 (0.017)	21 (0.188)	0.87	1.04 (0.63-1.72)
17-40	188 (0.683)	81 (0.295)	6 (0.220)	93 (0.169)	0.55	0.92 (0.70-1.21)
> 40	107 (0.652)	49 (0.299)	8 (0.049)	65 (0.198)	0.50	1.12 (0.81-1.53)
Disease location						
Colonic	131 (0.633)	68 (0.328)	8 (0.039)	84 (0.203)	0.35	1.15 (0.86-1.54)
Ileocaecal	81 (0.653)	37 (0.298)	6 (0.048)	49 (0.198)	0.56	1.11 (0.78-1.58)
Ileal	115 (0.719)	44 (0.275)	1 (0.006)	46 (0.144)	0.12	0.76 (0.53-1.08)
Disease behaviour						
Non-stricturing/non-penetrating	181 (0.644)	92 (0.327)	8 (0.028)	108 (0.192)	0.60	1.07 (0.82-1.40)
Penetrating	37 (0.649)	19 (0.333)	1 (0.018)	21 (0.184)	0.94	1.02 (0.62-1.68)
Stricturing	113 (0.720)	38 (0.242)	6 (0.038)	50 (0.159)	0.37	0.86 (0.61-1.21)

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

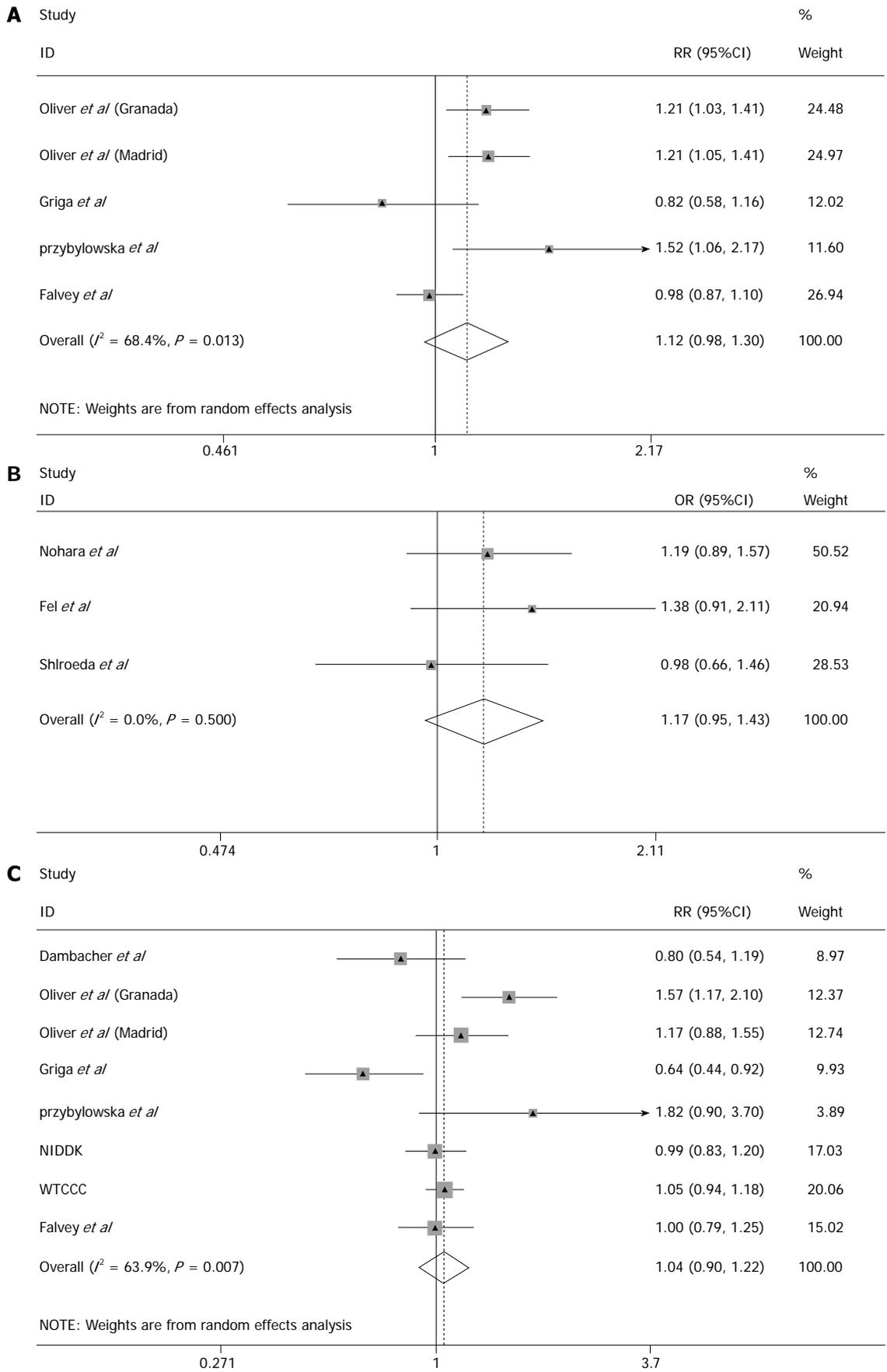
**Table 4 Allele and genotype frequencies of MIF CATT5-8 haplotypes in New Zealand Caucasian controls and inflammatory bowel disease patients**

Phenotype	Genotype <sup>a</sup>			MAF	Allelic <i>P</i> (unadjusted)	OR (95%CI)
	5/5	5/X	X/X			
Control	27 (0.050)	187 (0.350)	321 (0.600)	241 (0.225)		
IBD	54 (0.055)	366 (0.375)	555 (0.569)	474 (0.243)	0.27	1.10 (0.93-1.32)
UC	24 (0.051)	172 (0.364)	277 (0.586)	220 (0.233)	0.70	1.04 (0.85-1.28)
Age at first diagnosis (yr)						
< 17	0	11 (0.393)	17 (0.607)	11 (0.196)	0.61	1.04 (0.43-1.65)
17-40	10 (0.043)	91 (0.394)	130 (0.563)	111 (0.240)	0.52	1.09 (0.84-1.41)
> 40	14 (0.065)	70 (0.327)	130 (0.607)	98 (0.229)	0.88	1.02 (0.78-1.33)
Disease location						
Proctitis	12 (0.073)	56 (0.341)	96 (0.585)	80 (0.244)	0.48	1.11 (0.83-1.48)
Left-sided	5 (0.040)	48 (0.387)	71 (0.573)	58 (0.234)	0.77	1.05 (0.76-1.46)
Extensive	7 (0.039)	67 (0.372)	106 (0.589)	81 (0.225)	0.99	1.00 (0.75-1.33)
CD	30 (0.063)	182 (0.555)	264 (0.555)	242 (0.254)	0.12	1.18 (0.96-1.44)
Age at first diagnosis (yr)						
< 17	4 (0.073)	22 (0.400)	29 (0.527)	30 (0.273)	0.26	1.29 (0.83-2.01)
17-40	15 (0.057)	104 (0.394)	145 (0.549)	134 (0.254)	0.21	1.17 (0.92-1.49)
> 40	11 (0.070)	56 (0.357)	90 (0.573)	78 (0.248)	0.39	1.14 (0.85-1.52)
Disease location						
Colonic	14 (0.070)	75 (0.375)	111 (0.555)	103 (0.258)	0.19	1.19 (0.91-1.56)
Ileocaecal	7 (0.058)	48 (0.397)	66 (0.545)	62 (0.256)	0.19	1.19 (0.86-1.64)
Ileal	9 (0.060)	56 (0.371)	86 (0.570)	74 (0.245)	0.47	1.12 (0.83-1.51)
Disease behaviour						
Non-stricturing/non-penetrating	18 (0.066)	104 (0.382)	150 (0.551)	140 (0.257)	0.15	1.19 (0.94-1.51)
Penetrating	1 (0.019)	20 (0.377)	32 (0.604)	22 (0.208)	0.68	0.90 (0.55-1.47)
Stricturing	11 (0.073)	58 (0.384)	82 (0.543)	80 (0.265)	0.15	1.24 (0.93-1.66)

<sup>a</sup>X = 6, 7, or 8 CATT repeats. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

dataset<sup>[18]</sup>. With respect to the length polymorphism, *MIF* CATT<sub>5</sub>/CATT<sub>5</sub> genotype has been found to confer a

protective effect against IBD in Japanese individuals aged over 20 years (OR = 0.33, 95%CI: 0.14-0.82. *P* = 0.013)<sup>[18]</sup>,



**Figure 1 Meta-analyses of migration inhibitory factor-173G > C in Caucasian and East Asian inflammatory bowel disease datasets.** Meta-analyses of migration inhibitory factor (MIF)-173G > C were performed by the Mantel-Haenszel method using a random effects model. On each Forest plot the 95%CI of the individual datasets are represented by horizontal lines and the total number of study participants in each dataset is proportional to the size of the square. The diamond represents the pooled OR with 95%CI delineated by the diamond's width. A: The combined Caucasian ulcerative colitis (UC) dataset; B: The combined East Asian UC datasets; C: The combined Caucasian Crohn's disease dataset had 100% power to detect an effect size of OR = 1.40 at  $\alpha = 0.05$ .

whilst the CATT<sub>7</sub>/CATT<sub>7</sub> genotype was significantly associated with both a chronic continuous phenotype (OR = 5.49, 95%CI: 1.19-25.3,  $P = 0.015$ ) and distal disease location (OR = 6.10, 95%CI: 1.32-28.3,  $P = 0.0091$ ) in Japanese subjects. However, no association of CATT repeat length with UC risk was observed in two Spanish datasets<sup>[16]</sup>.

The story is similar for CD. Of the previous studies to have investigated the association between *MIF*-173G > C and CD, four found no association<sup>[12,13,16,17]</sup>, and one reported a protective effect of the minor allele, *MIF*-173C<sup>[14,16]</sup>. Furthermore, in one dataset, despite no association with overall CD susceptibility, further analysis revealed *MIF*-173C influenced CD phenotype; conferring a protective effect against the development of upper GI CD (OR = 0.31, 95%CI: 0.118-0.789,  $P = 0.01$ )<sup>[12]</sup>.

### *MIF* promoter polymorphisms in New Zealand Caucasians

Our study sought to resolve the discordance observed between previous investigations by conducting the single largest Caucasian dataset (1006 New Zealand cases, 540 New Zealand controls) study of *MIF* promoter polymorphisms, and employing meta-analysis. Single-site analysis found no evidence of association of *MIF* promoter polymorphisms with overall IBD susceptibility in New Zealand Caucasians. Haplotype analysis found preliminary evidence that a rare haplotype, CATT<sub>5</sub>-173C, which is the least active haplotype *in vitro*<sup>[9]</sup>, confers protection against IBD (OR = 0.22, 95%CI: 0.05-0.99,  $P_{unadjusted} = 0.03$ ). However, as the functional effects of *MIF* promoter polymorphisms are cell type specific and relevant experiments in monocytes, the primary cell type responsible for MIF dependent mucosal inflammation in IBD are lacking, no firm conclusions can be drawn from these observations<sup>[5]</sup>.

### Meta-analyses of *MIF*-173G > C in Caucasians and East Asians

Subsequent meta-analyses with additional Caucasian CD (3373 patients, 5543 controls), Caucasian UC (794 patients, 1499 controls), and Asian UC (416 patients, 789 controls) datasets also detected no association of *MIF*-173G > C with CD or UC in Caucasians, or with UC in East Asians (Figure 1). However, our Caucasian meta-analysis is limited by the presence of significant heterogeneity among the datasets, thus it is not possible to completely rule out the possibility that *MIF* promoter polymorphism may alter susceptibility to IBD in some Caucasian populations. In contrast, no significant heterogeneity was detected in the East Asian datasets ( $P_{het} = 0.50$ ), therefore the discordance previously observed is likely to be due to sample size rather than to differences in baseline characteristics of the study participants.

In conclusion, based on the results of our study, neither *MIF*-173G > C nor *MIF* CATT<sub>5-8</sub> are risk factors for IBD in New Zealand Caucasians, nor is *MIF*-173G > C a risk factor for UC in East Asians. The results of meta-

analysis do not support *MIF*-173G > C as a susceptibility factor for IBD in Caucasian datasets however, because significant heterogeneity exists between the populations investigated, the possibility that a significant effect exists in a subgroup of these populations cannot be excluded. Additional well-powered studies in European Caucasian datasets would be required to clarify this point.

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## COMMENTS

### Background

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine implicated in the pathophysiology of inflammatory arthritis, systemic lupus erythematosus, atherosclerosis, and cancer. Evidence from animal models and human inflammatory bowel disease (IBD) indicates that MIF is also an important mediator of IBD; however few studies have considered its mechanism of action in these diseases. In recent years, evidence regarding the role of MIF in inflammatory disease has accumulated rapidly, and efforts have subsequently turned to the development of specific anti-MIF therapies for use in human disease.

### Research frontiers

An international phase one trial of a neutralizing anti-MIF antibody is currently underway for lupus nephritis. On this background authors believe that critical appraisal of the role of MIF in IBD is needed in order for IBD patients to benefit from developments in this field. Two functional variants of the *MIF* promoter region have been identified. Despite clear association with other inflammatory diseases, the contribution of these polymorphisms to IBD risk and phenotype remains uncertain due to previous studies being under-powered.

### Innovations and breakthroughs

This paper describes the largest single cohort study of *MIF* promoter polymorphisms to date, as well as meta-analysis of all available genotype data on Caucasians and East Asians. Their population based cohort study had 93% power, and the meta-analyses 100% power, to detect an effect size of OR = 1.40 ( $\alpha = 0.05$ ). They found no evidence of association of *MIF* promoter polymorphisms with IBD.

### Applications

This study is important as it provides clear evidence that genetic variants of MIF

do not influence IBD susceptibility. Their findings are in agreement with recent research that indicates the contribution of MIF to IBD pathophysiology is not determined at the level of nuclear transcription.

### Peer review

The role of MIF in the pathogenesis of IBD has been intensively discussed during last couple of years, including the promoter polymorphism of this gene. It is also seen as one of the possible novel therapeutic targets for the management of IBD. The group has contributed to the research in this field by contributing data about the prevalence of the MIF promoter polymorphism in large population of patients and healthy controls. Furthermore, they have meta-analyzed this new data with previously published data, and concluded that there is no evidence of association of *MIF* promoter polymorphisms with IBD. Paper is well structured, comprehensive and clearly written.

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## Effects of entecavir and lamivudine for hepatitis B decompensated cirrhosis: Meta-analysis

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### Abstract

**AIM:** To compare the effects of entecavir (ETV) and lamivudine (LAM) for the treatment of hepatitis B decompensated cirrhosis using a meta-analysis.

**METHODS:** We conducted a literature search for all eligible studies published prior to May 30, 2013 using PUBMED, MEDLINE, EMBASE, the China National Knowledge Infrastructure (CNKI), the VIP database, the Wanfang database and the Cochrane Controlled Trial Register. Randomized controlled trials (RCTs) comparing ETV with LAM for the treatment of hepatitis B decompensated cirrhosis were included. The data were analyzed with Review Manager Software 5.0.2. We used RR as an effect measure, and reported its 95%CI. The meta-analysis was performed using either a fixed-effect or random-effect model, based on the absence or presence of significant heterogeneity. Two reviewers assessed the risk of bias and extracted data independently and in duplicate. The analysis was executed using the main outcome parameters including

hepatitis B virus (HBV) DNA undetectability, HBV DNA level, hepatitis B e antigen (HBeAg) seroconversion, alanine aminotransferase (ALT) level, albumin level, total bilirubin (TBIL) level, prothrombin time activity (PTA) level, Child-Turcotte-Pugh (CTP) score, mortality, drug-resistance, and adverse reactions. Meta-analysis of the included trials and subgroup analyses were conducted to examine the association between pre-specified characteristics and the therapeutic effects of the two agents.

**RESULTS:** Thirteen eligible trials (873 patients in total) were included and evaluated for methodological quality and heterogeneity. Of these studies, all had baseline comparability, 12 of them reported baseline values of the two treatment groups in detail. Following various treatment durations (12, 24, 36, 48 and > 48 wk), both ETV and LAM significantly reduced HBV DNA level, however, reductions were greater in the ETV group (MD = -0.66, 95%CI: -0.83-0.50,  $P < 0.00001$ ), (MD = -0.93, 95%CI: -1.36-0.51,  $P < 0.00001$ ), (MD = -1.4, 95%CI: -1.78-1.01,  $P < 0.00001$ ), (MD = -1.18, 95%CI: -1.90-0.46,  $P = 0.001$ ), (MD = -0.14, 95%CI: -0.17-0.11,  $P < 0.00001$ , respectively). At 12, 24 and 48 wk of treatment, ETV had a significant effect on the rate of HBV DNA undetectability (RR = 1.55, 95%CI: 1.22-1.99,  $P = 0.0004$ ), (RR = 1.25, 95%CI: 1.13-1.38,  $P < 0.0001$ ), (RR = 1.2, 95%CI: 1.10-1.32,  $P < 0.0001$ , respectively). Although HBeAg seroconversion in the ETV group was more pronounced than that in the LAM group at 24 wk (27.90% vs 26.19%) and 48 wk (31.52% vs 25.00%) of treatment, there was no statistically significant difference between them (RR = 1.49, 95%CI: 0.98-2.28,  $P = 0.07$ ), (RR = 1.27, 95%CI: 0.98-1.65,  $P = 0.07$ , respectively). Following various treatment durations, both the ETV group and the LAM group showed significantly improved liver function (ALT, AIB, TBIL, PTA and CTP levels) and reduced mortality (ETV 6.37%, LAM 7.89%). The effects in the ETV group (0.33%) were statistically lower than those in the LAM group (14.33%) regarding the rate of drug-resistance (RR

= 0.1, 95%CI: 0.04-0.24,  $P \leq 0.00001$ ). In addition, no severe adverse reactions were observed in the two treatment groups.

**CONCLUSION:** ETV and LAM significantly improved liver function and reduced mortality. Both drugs produced similar serological responses, and were safe and well tolerated. However, ETV resulted in a better virological response and lower drug-resistance, but is more expensive.

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**Key words:** Hepatitis B; Decompensated cirrhosis; Entecavir; Lamivudine; Randomized controlled trial; Meta-analysis.

**Core tip:** This meta-analysis was conducted to compare the effects of entecavir (ETV) and lamivudine (LAM) in the treatment of hepatitis B associated decompensated cirrhosis. The results suggested that ETV and LAM significantly improved liver function and reduced mortality. Both drugs produced similar serological responses, and were safe and well tolerated. However, LAM had higher drug-resistance and is therefore unsuitable for the long-term treatment of patients with hepatitis B decompensated cirrhosis. ETV can be used as the first-line drug for long-term treatment of patients with hepatitis B decompensated cirrhosis as it has stronger anti-viral activity and extremely low drug-resistance.

Ye XG, Su QM. Effects of entecavir and lamivudine for hepatitis B decompensated cirrhosis: Meta-analysis. *World J Gastroenterol* 2013; 19(39): 6665-6678 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i39/6665.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6665>

## INTRODUCTION

The treatment of chronic hepatitis B (CHB) is a major healthcare problem affecting over 350 million people worldwide<sup>[1]</sup>. Approximately 25%-40% of infected patients will develop various life-threatening conditions such as liver failure, liver cirrhosis (LC) and hepatocellular carcinoma (HCC). The 5-year survival rate is 84% in patients with compensated cirrhosis, but decreases to 14%-35% in individuals with decompensated cirrhosis<sup>[2]</sup>. Antiviral therapy is now considered to be the most important measure to prevent further development of this disease. Rapid and effective antiviral therapy can not only improve liver function and clinical symptoms as well as postpone progression to LC, but can also reverse the process of LC, prolong survival time and improve the quality of life. Some researchers have shown that mortality due to hepatitis B virus (HBV) and LC was positively correlated<sup>[3,4]</sup>. As interferon is prohibited for the treatment of decompensated cirrhosis, nucleosides

or nucleoside analogues have become the primary drugs for antiviral therapy. Entecavir (ETV) is currently the strongest nucleoside analogue and the first-line drug for hepatitis B. It has the advantages of low drug-resistance and high safety, thus it is suitable for long-term use. However, due to its higher cost, the long-term use of ETV results in heavy financial pressures for patients with hepatitis B decompensated cirrhosis and their families. Lamivudine (LAM) is a moderate strength nucleoside analogue, and has high resistance following long-term use, which leads to treatment failure. However, due to its lower cost, LAM has a pharmacoeconomic advantage. Although there have many studies conducted on the efficacy of ETV compared with LAM for the treatment of patients with hepatitis B decompensated cirrhosis, there are few systematic reviews on this topic<sup>[5]</sup>. The roles of the two drugs in hepatitis B decompensated cirrhosis are not yet completely clear. Therefore, we conducted a meta-analysis of randomized controlled trials (RCTs) using the Cochrane methodology and explored the efficacy of ETV compared with LAM in patients with hepatitis B decompensated cirrhosis.

## MATERIALS AND METHODS

### Literature search

We searched PUBMED, MEDLINE, EMBASE, CNKI (China National Knowledge Infrastructure), the VIP database, the Wanfang database and the Cochrane Controlled Trial Register for the relevant studies published up to May 30, 2013. The following keywords were used for the search: "hepatitis B", "decompensated cirrhosis", "entecavir", "lamivudine", and "RCTs". The reference lists of eligible studies were also searched. The language of the trials was not limited.

### Inclusion criteria

The following inclusion criteria were used: (1) RCTs; (2) Articles studying hepatitis B decompensated cirrhosis patients, who were included in Chinese articles according to the diagnostic standards of the China guidelines for HBV management (2005)<sup>[6]</sup>, in foreign articles diagnosis was based on clinical, biochemical, radiological and histological responses, and a Child-Turcotte-Pugh (CTP) score  $\geq 7$ ; (3) Studies comparing the treatment methods of ETV (0.5 mg/d) and LAM (100 mg/d). Both groups were given symptomatic treatment and conventional treatment; and (4) The main outcome parameters included the rate of HBV DNA undetectability, HBV DNA level, hepatitis B e antigen (HBeAg) seroconversion, alanine aminotransferase (ALT) levels, albumin (ALB) levels, total bilirubin (TBIL) levels, prothrombin time activity (PTA) levels, CTP score, mortality, drug-resistance, and adverse reactions.

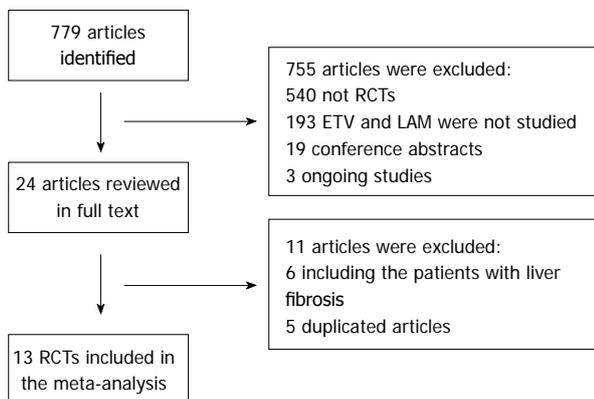
### Exclusion criteria

The following exclusion criteria were used: (1) Non-RCTs; (2) Insufficient analytical information regarding

**Table 1** Characteristics of included randomized controlled trials

Trial	Sample size (n)		mean age (yr)		Regimen		Duration (wk)	Observation time (wk)	Outcome parameters	Jadad scores
	ETV	LAM	ETV	LAM	ETV	LAM				
Feng <i>et al</i> <sup>[8]</sup>	22	25	-	-	0.5 mg/d	100 mg/d	48	4, 12, 24, 36, 48	ACDGI	3
Yang <i>et al</i> <sup>[9]</sup>	30	30	47.5 ± 9.7		0.5 mg/d	100 mg/d	48	4, 8, 12, 24, 48	ABCI	3
Shen <sup>[10]</sup>	40	40	46.5	48.5	0.5 mg/d	100 mg/d	48	48	BDEFIJ	3
Huang <i>et al</i> <sup>[11]</sup>	22	22	48.2	47.5	0.5 mg/d	100 mg/d	52	52	BDEFGHIJ	3
Chen <i>et al</i> <sup>[12]</sup>	23	24	48.5		0.5 mg/d	100 mg/d	48	24, 48	ACJ	3
Li <i>et al</i> <sup>[13]</sup>	40	40	51.0		0.5 mg/d	100 mg/d	48	12, 24, 48	ACDEFHI	2
Shao <i>et al</i> <sup>[14]</sup>	29	28	43.1 ± 10.262	44.11 ± 10.322	0.5 mg/d	100 mg/d	96	12, 24, 36, 48, 60, 72, 84, 96	AHIJ	3
Kong <sup>[15]</sup>	24	24	47.5		0.5 mg/d	100 mg/d	48	48	BDEFGH	2
Hyun <i>et al</i> <sup>[16]</sup>	45	41	54 ± 9.4	53.7 ± 12.1	0.5 mg/d	100 mg/d	48	12, 24, 36, 48	ABCHIJ	3
Wang <i>et al</i> <sup>[17]</sup>	66	64	52.3 ± 15.8	50.8 ± 15.4	0.5 mg/d	100 mg/d	48	12, 24, 36, 48	ABCGHI	2
Yang <i>et al</i> <sup>[18]</sup>	32	42	47.8 ± 10.2		0.5 mg/d	100 mg/d	48	12, 24, 36, 48	ACDEFI	3
Zhou <i>et al</i> <sup>[19]</sup>	40	40	46 ± 14		0.5 mg/d	100 mg/d	48	12, 24, 48	BDH	3
Liu <i>et al</i> <sup>[20]</sup>	30	30	46.04 ± 10.79	45.75 ± 10.26	0.5 mg/d	100 mg/d	48	48	B	3

A: Hepatitis B virus (HBV) DNA undetectability; B: HBV DNA levels; C: Hepatitis B e antigen seroconversion; D: Alanine aminotransferase levels; E: Albumin levels; F: Total bilirubin levels; G: Prothrombin time activity levels; H: Child-Turcotte-Pugh score; I: Drug-resistance; J: Mortality. ETV: Effects of entecavir; LAM: Lamivudine.



**Figure 1** Flow diagram of the randomized controlled trials reviewed. RCT: Randomized controlled trial; ETV: Entecavir; LAM: Lamivudine.

treatment schedule, follow-up, and outcomes; (3) Receiving interferon, nucleosides or nucleotides for CHB within 6 mo of the trial; (4) Coinfection with hepatitis A, C, D, E virus, cytomegalovirus, or HIV; and (5) Patients with liver failure, HCC, and liver-related complications caused by alcoholism, autoimmune disease, and cholestasis.

### Data extraction

Data extraction was assessed independently by two reviewers (Song LY and Zhang SR). Discrepancies among reviewers were resolved by discussions between the reviewers or by a third person (Ou-Yang RJ). Basic information obtained from each eligible trial included the study design (randomization, allocation concealment, blinding method, description of withdrawals and dropouts), patient characteristics, numbers in each group, related study results and treatment duration. Data were reviewed to eliminate duplicate reports of the same trial.

### Statistical analysis

We used Review Manager Software 5.0.2 (Cochrane Col-

laboration, Oxford, United Kingdom) to carry out data analysis. We used RR as an effect measure for dichotomous data, mean difference (MD) as an effect measure for continuous data, and reported their 95%CI. The meta-analysis was performed using a fixed-effect or random-effect model, based on the absence or presence of significant heterogeneity.

Statistical heterogeneity between trials was evaluated by  $\chi^2$  and  $I^2$  analysis. The fixed-effect method was used in the absence of statistically significant heterogeneity ( $P \geq 0.1$ ), the random-effect method was used when the heterogeneity test was statistically significant ( $P < 0.1$ ). A value of  $P < 0.05$  was regarded as statistically significant. We used subgroup analyses to examine the association of pre-specified characteristics (treatment duration) with treatment effect, sensitivity analysis was used to estimate the stability of the results, and funnel plots were used to assess publication bias if more than five trials were included<sup>[7]</sup>.

## RESULTS

### Characteristics and quality of studies

The process of identifying the included trials is presented in Figure 1. We initially identified 779 abstracts, and after evaluating the full texts, we included 13 trials (12 in Chinese and 1 in English) based on the pre-specified criteria. A total of 873 patients were included in the study: 423 treated with ETV and 450 treated with LAM. Table 1 shows the characteristics of the 13 trials. Of these studies, all showed baseline comparability, 12 of them reported the baseline values of the two groups in detail, 1 only referred to the two groups as having no significant differences in gender, age and duration<sup>[12]</sup>. One described the method of randomization in detail<sup>[9]</sup>, 9 referred to randomization, but did not describe the method of randomization in detail<sup>[8,11-15,18-20]</sup>. None of the trials referred to allocation concealment and blinding method. Six described the reasons for withdrawals and dropouts<sup>[8,12-14,16]</sup>. Quality

assessment of the trials was performed with Jadad scores that ranged between 1 and 5<sup>[21]</sup>. Based on these scores, 10 trials were of high quality ( $\geq 3$  scores)<sup>[8-12,14,16,18-20]</sup>, and 3 trials were of inferior quality ( $< 3$  scores)<sup>[13,15,17]</sup>.

### HBV DNA undetectability

In this analysis, 8 trials reported rates of HBV DNA undetectability. According to  $\chi^2$  and  $I^2$  analyses, heterogeneity was observed ( $\chi^2 = 40.42$ ,  $P = 0.03$ ,  $I^2 = 38\%$ ); therefore, we used the random-effect method to analyze the data. At 12, 24 and 48 wk of treatment, the rate of HBV DNA undetectability was higher in the ETV group than in the LAM group, and the difference between the two groups was statistically significant [(RR = 1.55, 95%CI: 1.22-1.99,  $P = 0.0004$ ), (RR = 1.25, 95%CI: 1.13-1.38,  $P < 0.0001$ ), (RR = 1.2, 95%CI: 1.10-1.32,  $P < 0.0001$ , respectively)], while at 36 and  $> 48$  wk, the rate of HBV DNA undetectability between the two groups was similar, and no statistically significant differences were observed [(RR = 1.21, 95%CI: 0.92-1.59,  $P = 0.18$ ), (RR = 1.27, 95%CI: 0.98-1.64,  $P = 0.07$ ), respectively] (Figure 2A).

### HBV DNA levels

In this analysis, 8 trials reported HBV DNA levels. According to  $\chi^2$  and  $I^2$  analyses, heterogeneity was observed ( $\chi^2 = 1274.13$ ,  $P < 0.00001$ ,  $I^2 = 99\%$ ); therefore, we used the random-effect method to analyze the data. At 12, 24, 36, 48, and  $> 48$  wk, HBV DNA levels were lower in the ETV group than in the LAM group, and the difference between the two groups was statistically significant [(MD = -0.66, 95%CI: -0.83-0.50,  $P < 0.00001$ ), (MD = -0.93, 95%CI: 1.36-0.51,  $P < 0.0001$ ), (MD = -1.4, 95%CI: -1.78-1.01,  $P < 0.00001$ ), (MD = -1.18, 95%CI: -1.90-0.46,  $P = 0.001$ ), (MD = -0.14, 95%CI: -0.17-0.11,  $P < 0.00001$ ), respectively] (Figure 2B).

### HBeAg seroconversion

In this analysis, 7 trials reported the rate of HBeAg seroconversion. According to  $\chi^2$  and  $I^2$  analyses, heterogeneity was not observed ( $\chi^2 = 6.88$ ,  $P = 0.87$ ,  $I^2 = 0\%$ ); therefore, we used the fixed-effect method to analyze the data. At 12 wk, the rate of HBeAg seroconversion was higher in the ETV group than in the LAM group, and the difference between the two groups was statistically significant (RR = 2.05, 95%CI: 1.06-3.98,  $P = 0.03$ ), while at 24 and 48 wk, the rate of HBeAg seroconversion in the two groups was similar, and no statistically significant differences were observed. [(RR = 1.49, 95%CI: 0.98-2.28,  $P = 0.07$ ), (RR = 1.27, 95%CI: 0.98-1.65,  $P = 0.07$ ), respectively] (Figure 2C).

### ALT levels

In this analysis, 7 trials reported ALT levels. According to  $\chi^2$  and  $I^2$  analysis, heterogeneity was observed ( $\chi^2 = 110.78$ ,  $P < 0.00001$ ,  $I^2 = 89\%$ ); therefore, we used the random-effect method to analyze the data. At 48 wk, ALT levels were lower in the ETV group than in the LAM group, and the difference was statistically significant

(MD = -9.74, 95%CI: -17.87-1.61,  $P = 0.02$ ), while at 12, 24 and  $> 48$  wk, ALT levels in the two groups were similar, and no statistically significant differences were observed [(MD = -3.72, 95%CI: -8.7-1.26,  $P = 0.14$ ), (MD = -5.73, 95%CI: -15.52-4.06,  $P = 0.25$ ), (MD = -1.07, 95%CI: -15.73-13.59,  $P = 0.89$ ), respectively] (Figure 2D).

### ALB levels

In this analysis, 5 trials reported ALB levels. According to  $\chi^2$  and  $I^2$  analysis, heterogeneity was observed ( $\chi^2 = 89.49$ ,  $P < 0.00001$ ,  $I^2 = 91\%$ ); therefore, we used the random-effect method to analyze the data. At  $> 48$  wk, ALB levels were higher in the ETV group than in the LAM group, and the difference was statistically significant (MD = 1.84, 95%CI: -0.47-4.15,  $P = 0.0001$ ), while at 12, 24 and 48 wk, ALB levels in the two groups were similar, and no statistically significant differences were observed [(MD = -3.43, 95%CI: -14.3-7.45,  $P = 0.54$ ), (MD = 0.15, 95%CI: -0.94-1.25,  $P = 0.78$ ), (MD = 3.83, 95%CI: -0.11-7.77,  $P = 0.06$ ), respectively] (Figure 2E).

### TBIL levels

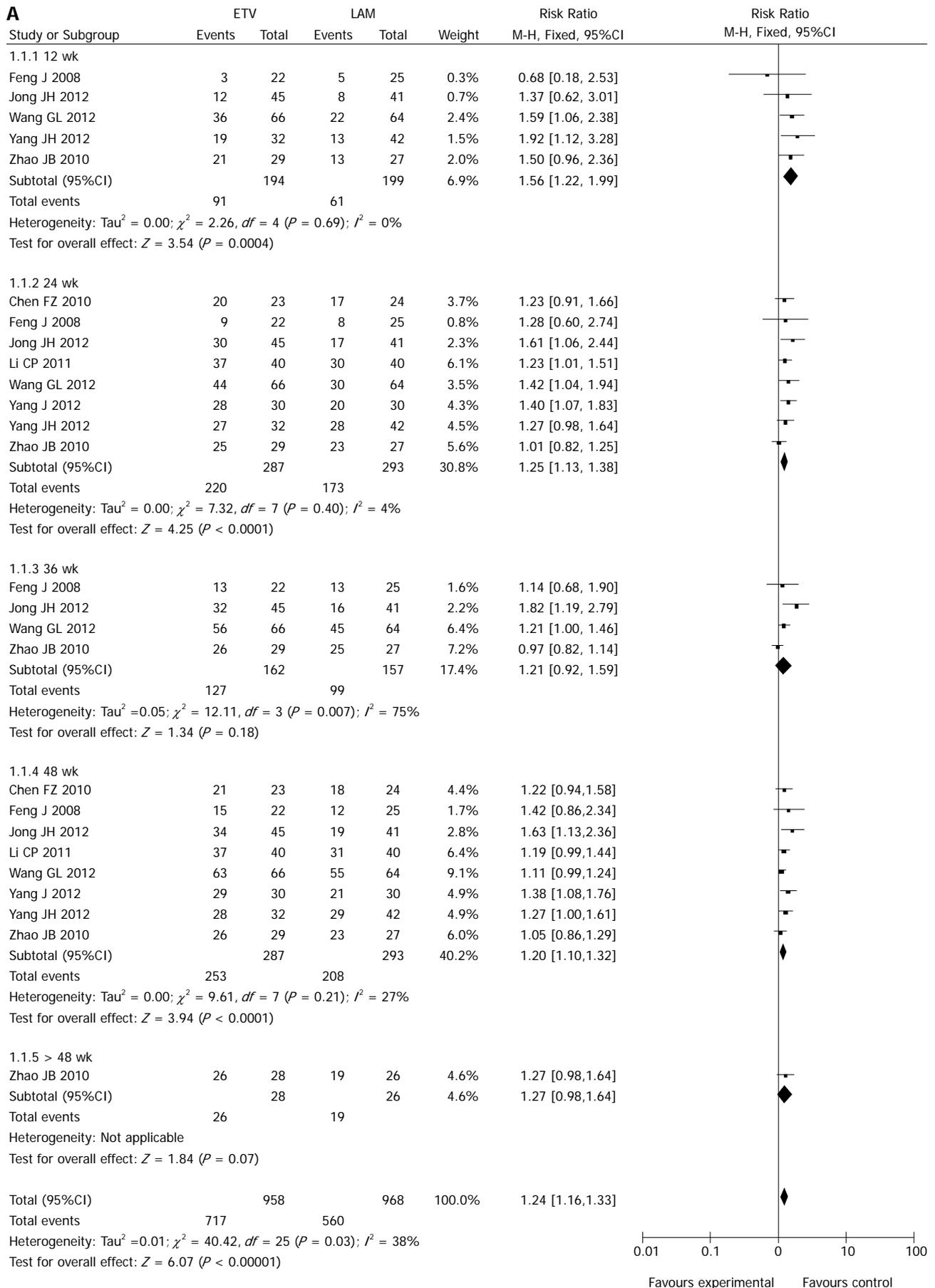
In this analysis, 5 trials reported TBIL levels. According to  $\chi^2$  and  $I^2$  analysis, heterogeneity was observed ( $\chi^2 = 29.21$ ,  $P \leq 0.00001$ ,  $I^2 = 90\%$ ); therefore, we used the random-effect method to analyze the data. At 12, 24 and 48 wk, TBIL levels were lower in the ETV group than in the LAM group, and the difference was statistically significant [(MD = -6.21, 95%CI: -8.86-3.57,  $P < 0.00001$ ), (MD = -6.61, 95%CI: -8.42-4.81,  $P < 0.00001$ ), (MD = -11.51, 95%CI: -17.18-5.84,  $P < 0.0001$ ), respectively], while at  $> 48$  wk, TBIL levels in the two groups were similar, and no statistically significant difference was observed (MD = -1.43, 95%CI: -10.52-7.66,  $P = 0.76$ ) (Figure 2F).

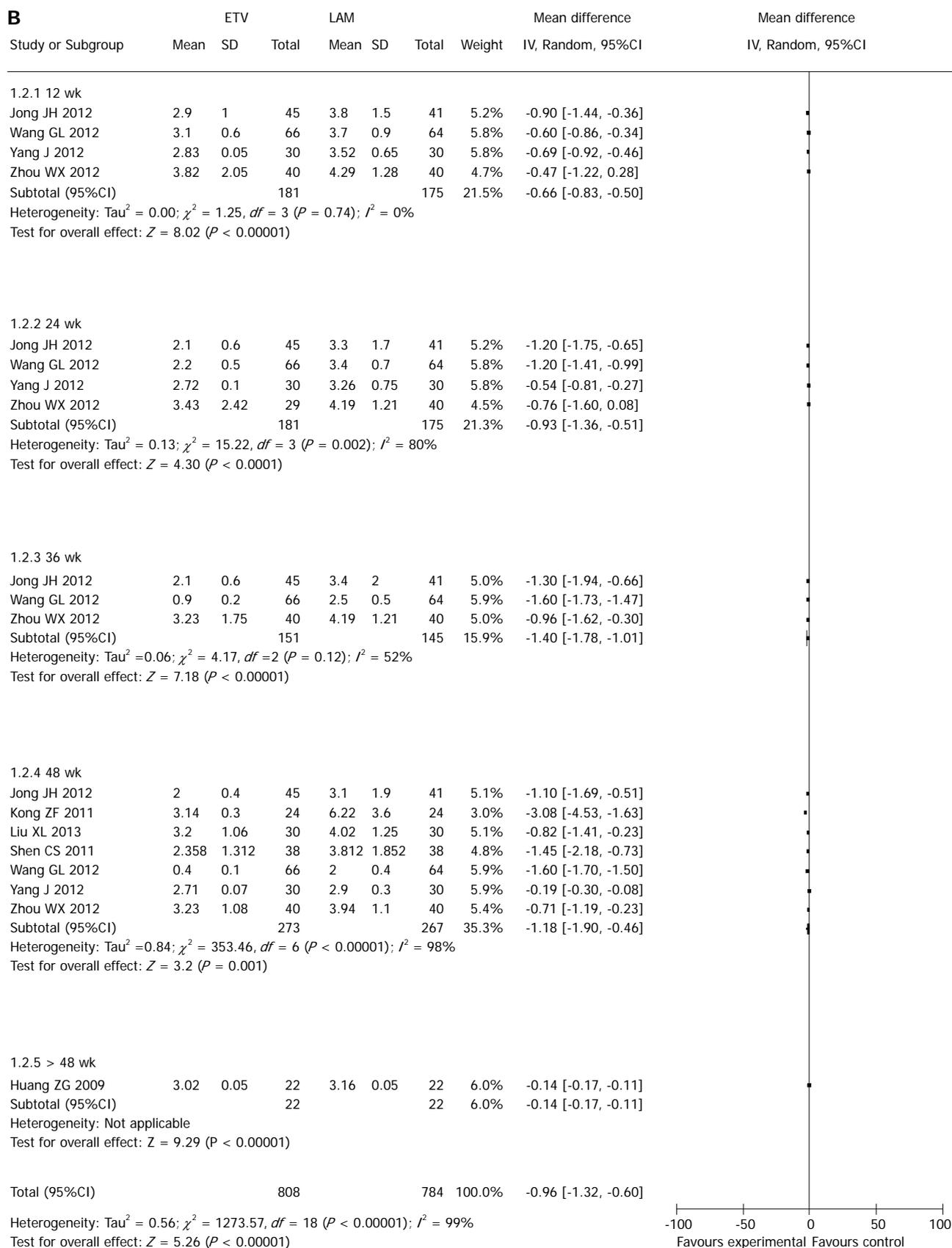
### PTA levels

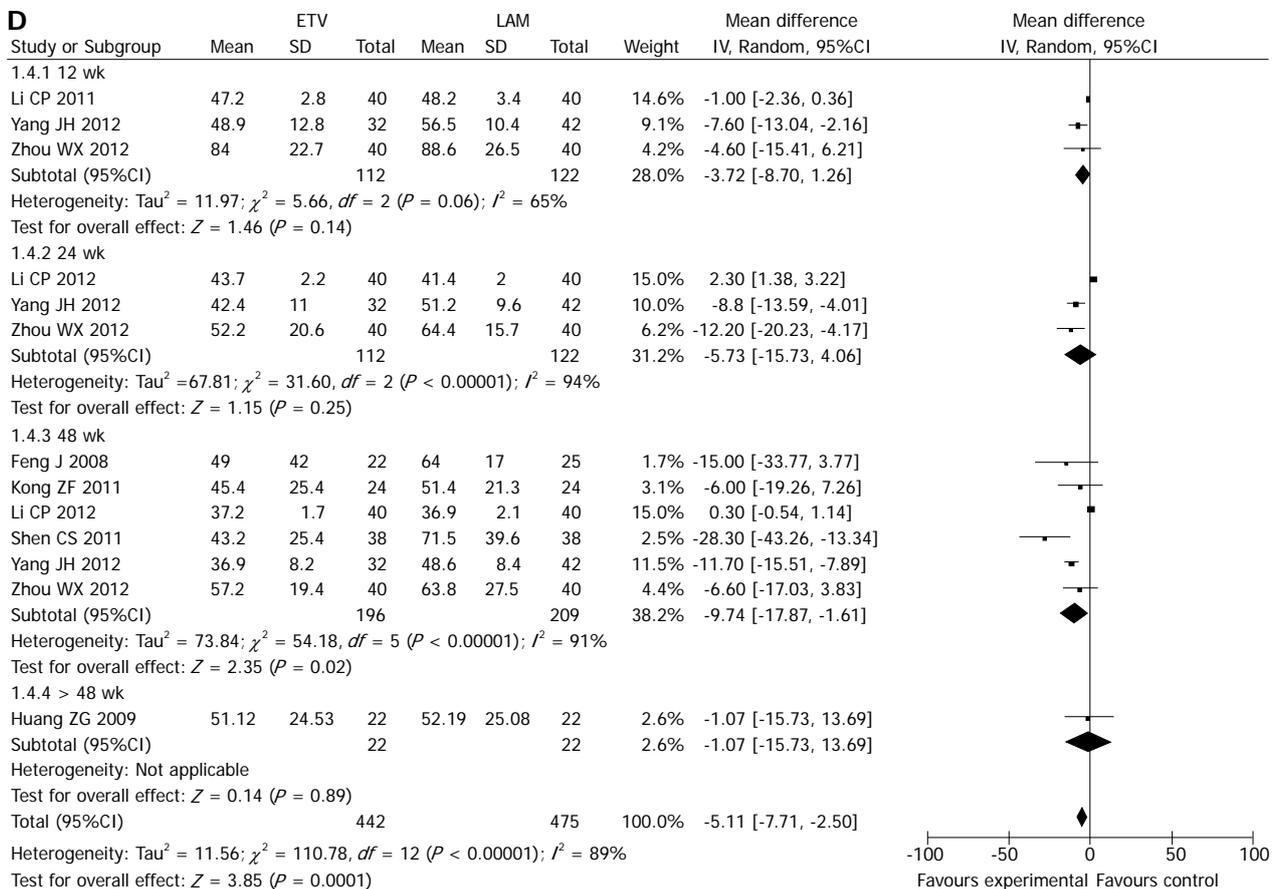
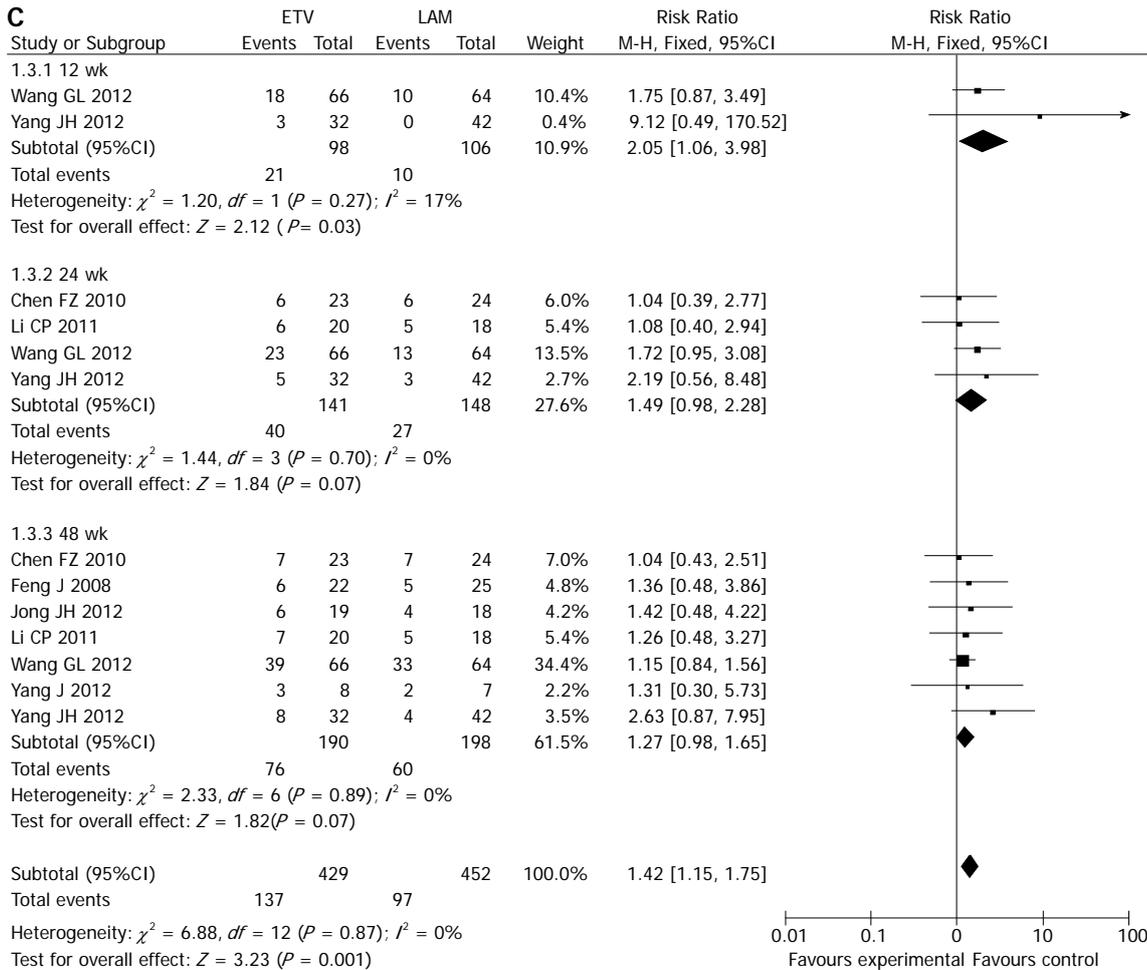
In this analysis, 4 trials reported PTA levels. According to  $\chi^2$  and  $I^2$  analysis, heterogeneity was observed ( $\chi^2 = 0.42$ ,  $P = 1.0$ ,  $I^2 = 0\%$ ); therefore, we used the fixed-effect method to analyze the data. At 12 and 24 wk, PTA levels were higher in the ETV group than in the LAM group, and the differences were statistically significant [(MD = 2, 95%CI: 0.26-3.74,  $P = 0.02$ ), (MD = 2.09, 95%CI: 0.29-3.88,  $P = 0.02$ ), respectively], while at 48 and  $> 48$  wk, PTA levels in the two groups were similar, and no statistically significant differences were observed [(MD = 1.68, 95%CI: -1.19-4.54,  $P = 0.25$ ), (MD = -0.40, 95%CI: -9.44-8.64,  $P = 0.93$ ), respectively] (Figure 2H).

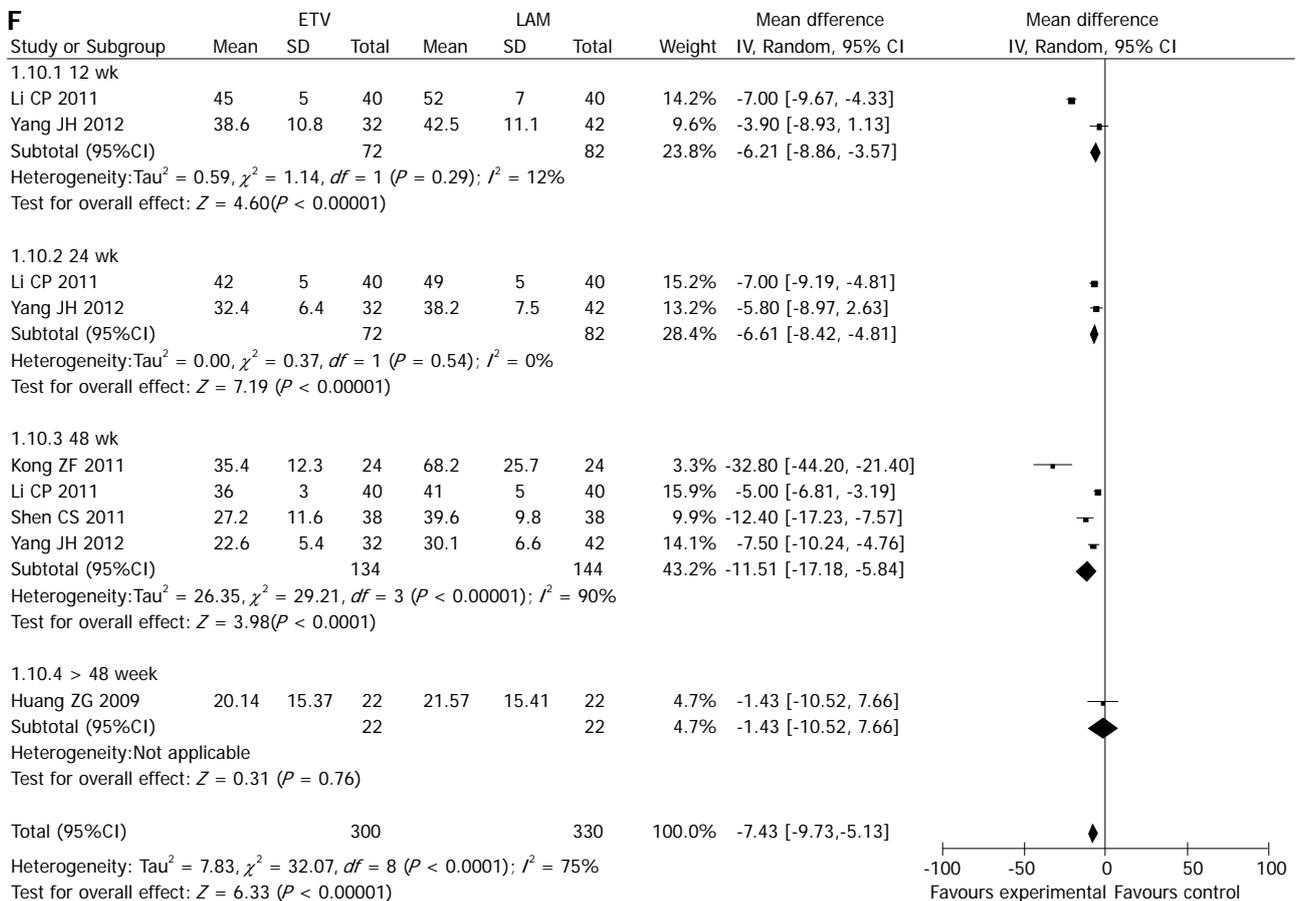
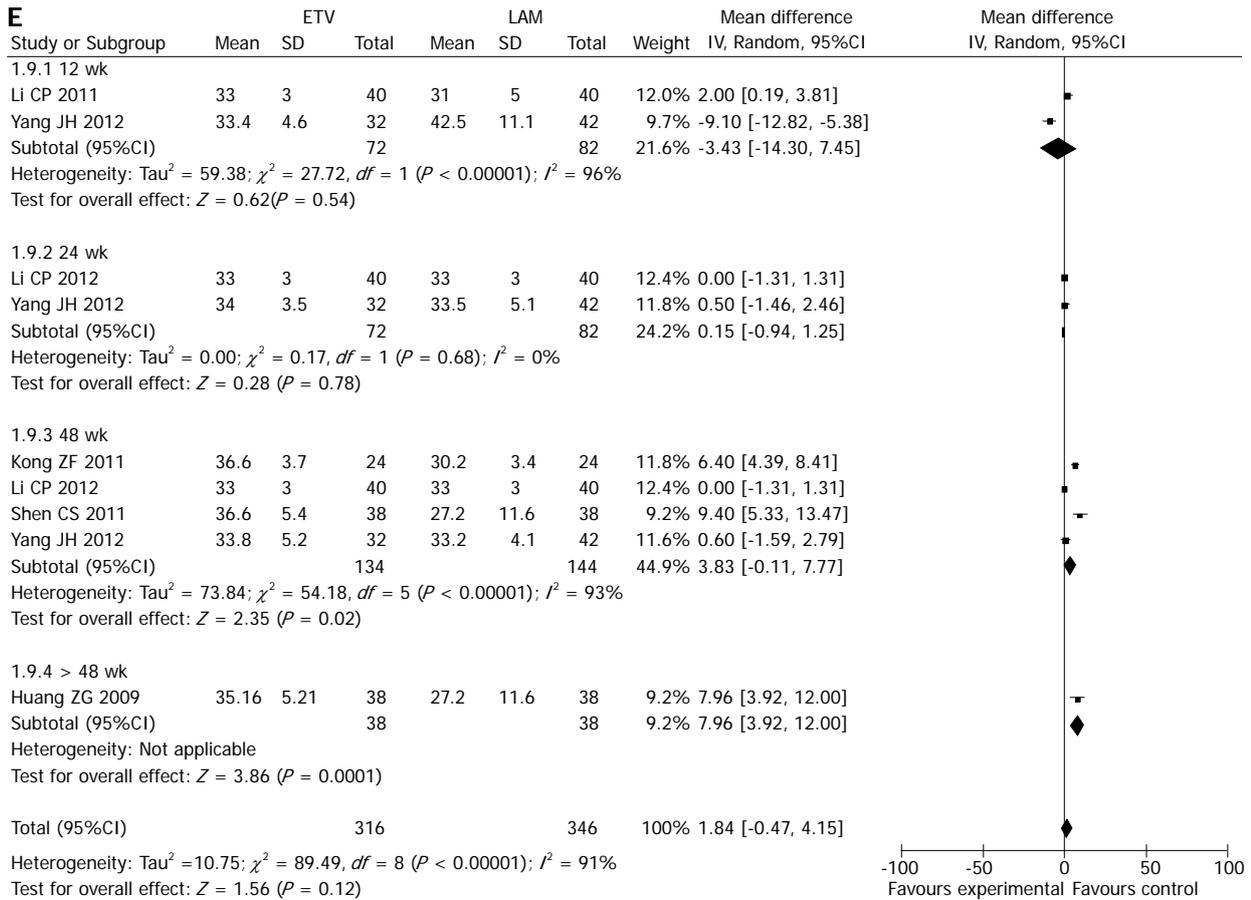
### CTP score

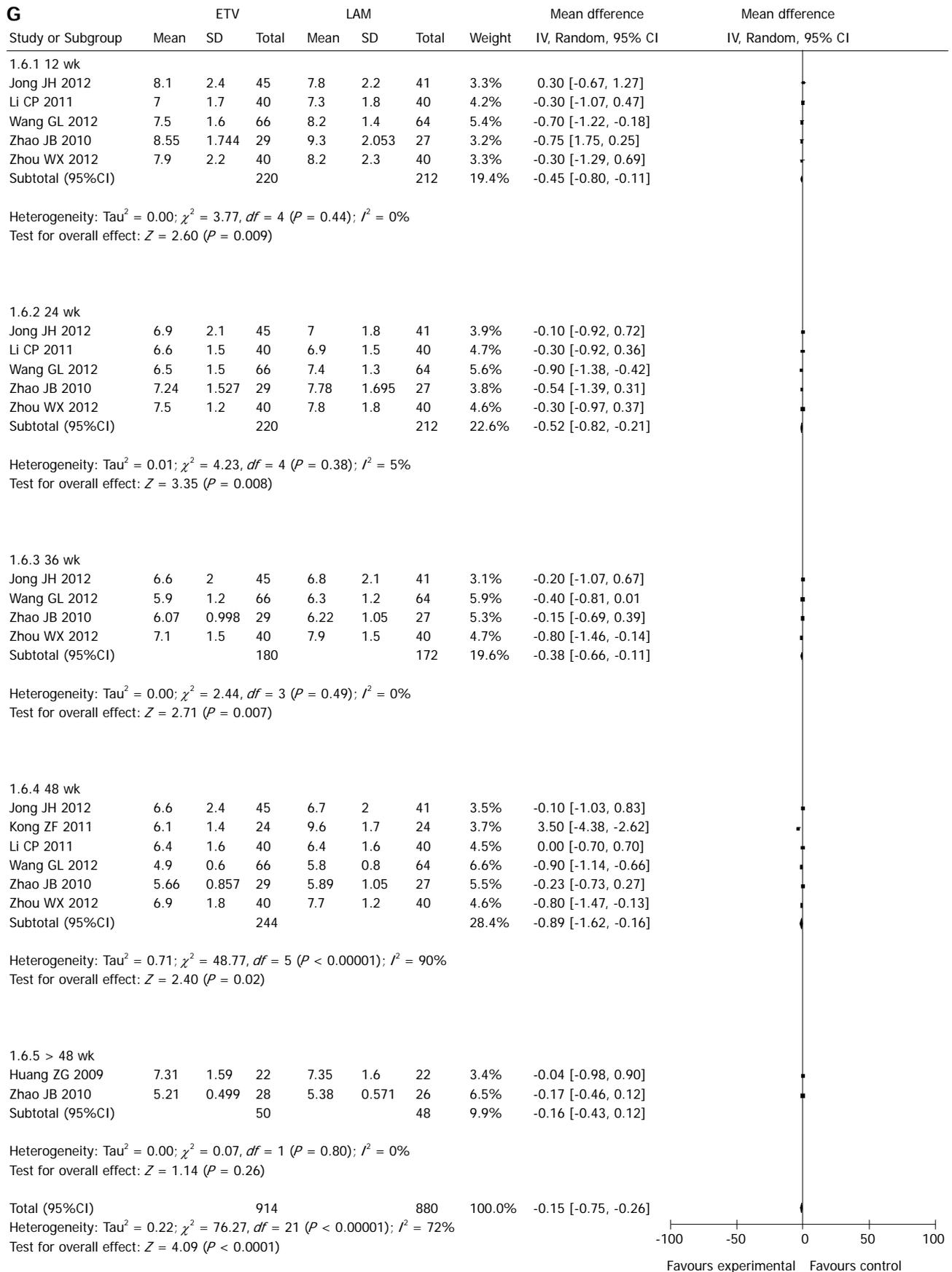
In this analysis, 7 trials reported the CTP score. According to  $\chi^2$  and  $I^2$  analysis, heterogeneity was observed ( $\chi^2 = 76.27$ ,  $P < 0.00001$ ,  $I^2 = 72\%$ ); therefore, we used the random-effect method to analyze the data. At 12, 24, 36 and 48 wk, the CTP score was lower in the ETV group than in the LAM group, and the differences were statistically significant [(MD = -0.45, 95%CI: -0.80-0.11,  $P =$

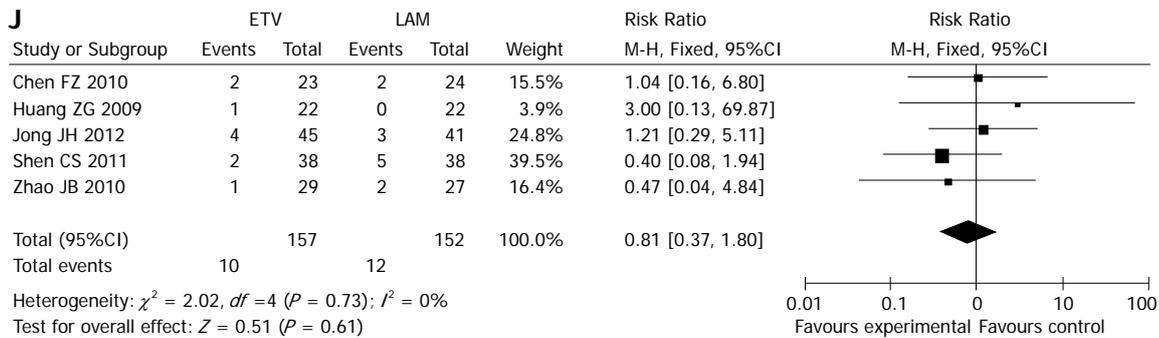
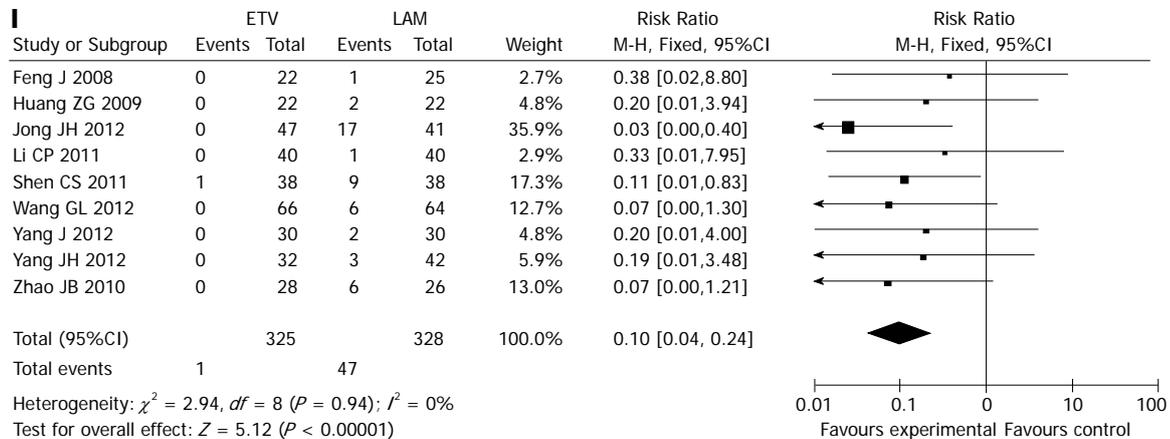
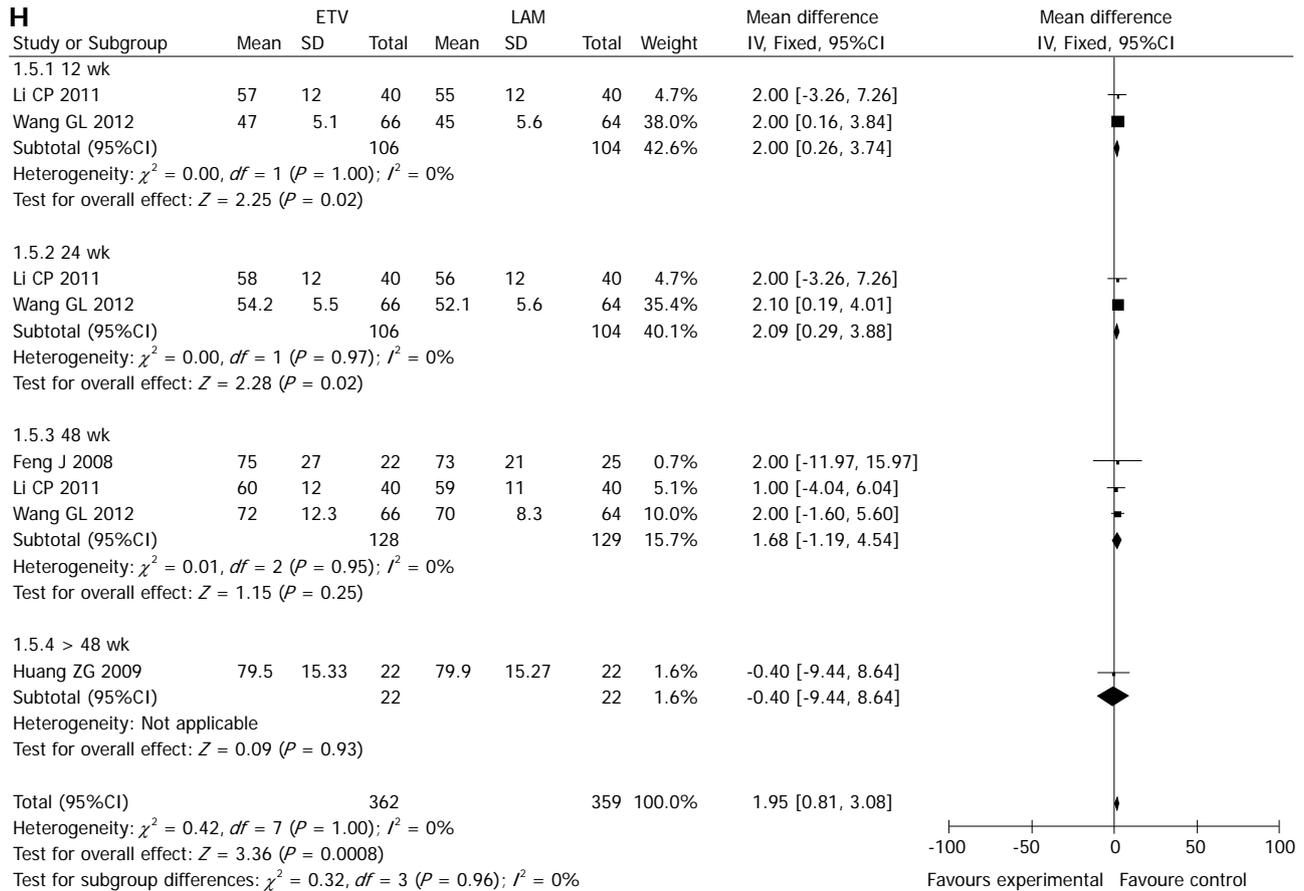


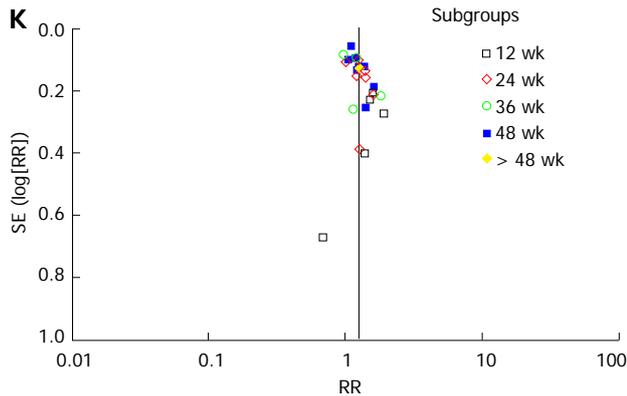












**Figure 2** Meta-analysis. A: Hepatitis B virus (HBV) DNA undetectability in the two treatment groups; B: HBV DNA levels in the two treatment groups; C: Hepatitis B e antigen (HBeAg) seroconversion in the two treatment groups; D: Alanine aminotransferase (ALT) levels in the two treatment groups; E: Albumin (ALB) levels in the two treatment groups; F: Total bilirubin (TBIL) levels in the two treatment groups; G: The child-Turcotte-Pugh (CTP) score in the two treatment groups; H: Prothrombin time activity (PTA) levels in the two treatment groups; I: Drug-resistance in the two treatment groups; J: Mortality in the two treatment groups; K: The two treatment groups included in the Randomized controlled trials (RCTs). ETV: Entecavir; LAM: Lamivudine.

**Table 2** Sensitivity analysis

Index	Total HBV DNA undetectability	
	RR	P value
Removing the inferior quality trials <sup>[13,15,17]</sup>	1.26 (1.15, 1.39)	< 0.0001
Using random-effect model	1.24 (1.16, 1.33)	< 0.00001
Using fixed-effect model	1.29 (1.22, 1.37)	< 0.00001

HBV: Hepatitis B virus.

0.009), (MD = -0.52, 95%CI: -0.82-0.21,  $P = 0.0008$ ), (MD = -0.38, 95%CI: -0.66-0.11,  $P = 0.007$ ), (MD = -0.89, 95%CI: -1.62-0.16,  $P = 0.02$ ), respectively], while at > 48 wk, the CTP score in the two groups was similar, and no statistically significant difference was observed (MD = -0.16, 95%CI: -0.43-0.12,  $P = 0.26$ ) (Figure 2G).

### Drug-resistance

In this analysis, 9 trials reported the rate of drug-resistance. According to  $\chi^2$  and  $I^2$  analysis, heterogeneity was not observed ( $\chi^2 = 2.94$ ,  $P = 0.94$ ,  $I^2 = 0\%$ ); therefore, we used the fixed-effect method to analyze the data. At the end of treatment, the rate of drug-resistance was lower in the ETV group (0.33%) than in the LAM group (14.33%), and the difference was statistically significant (RR = 0.1, 95%CI: 0.04-0.24,  $P \leq 0.00001$ ) (Figure 2I).

### Adverse reactions

In this analysis, 12 trials reported adverse reactions. The difference in adverse reactions between the two groups was not obvious. Patients in the two treatment groups did not experience severe adverse reactions, and common adverse reactions included headache, fatigue, nausea, diarrhea, hypersomnia, and insomnia. Five trials reported mortality. According to  $\chi^2$  and  $I^2$  analysis, heterogeneity was not observed ( $\chi^2 = 1.37$ ,  $P = 0.85$ ,  $I^2 = 0\%$ ); therefore, we used the fixed-effect method to analyze the data. At the end of treatment, the mortality rate in the two

groups (ETV 6.37% *vs* LAM 7.89%) was similar, and no statistically significant difference was observed (RR = 0.81, 95%CI: 0.37-1.80,  $P = 0.61$ ) (Figure 2J).

### Assessment of publication bias

We examined publication bias using a funnel plot. The results showed that the plot was funnel shaped which suggested the absence of significant publication bias (Figure 2K).

### Sensitivity analysis

Sensitivity analysis were performed by excluding certain studies. For example, when considering the rate of HBV DNA undetectability, using the fixed-effect model instead of the random-effect model, 3 inferior quality trials were removed. The ORs of all sensitivity analyses were larger than 1 and statistically significant ( $P < 0.05$ ), suggesting that the results of the meta-analysis were stable (Table 2).

## DISCUSSION

Nucleoside/nucleotide analogues (NUCs) are the only antiviral agents recommended for patients with hepatitis B decompensated cirrhosis<sup>[6]</sup>. As the first NUC used in the treatment of CHB, LAM has been widely used in the treatment of hepatitis B cirrhosis. A number of researchers have shown that LAM can effectively suppress HBV DNA replication and significantly improve liver function in patients with hepatitis B decompensated cirrhosis<sup>[22,23]</sup>. However, a critical weak point of LAM therapy is the frequent occurrence of resistant mutations and high drug-resistance in HBV<sup>[24]</sup>. As liver function in patients with LC is poor and progression of the disease is fast, the selection of appropriate drugs in the later period of treatment is difficult. ETV is a new cyclopentyl guanosine nucleoside analogue which is efficiently phosphorylated to its active triphosphate form by host cellular kinases. It blocks HBV replication by inhibiting HBV polymerase, the DNA strand *via* reverse transcription elongation, and

DNA-dependent plus-strand DNA synthesis<sup>[25]</sup>. ETV has the advantage of a higher rate of HBV DNA suppression, low drug-resistance and high safety, especially in LAM-resistant CHB patients<sup>[26]</sup>. Therefore, some researchers have attempted to use ETV for the treatment of hepatitis B decompensated cirrhosis<sup>[27,28]</sup>. However, it is more expensive than other nucleoside analogues.

In the present study, we included RCTs comparing ETV with LAM in patients with hepatitis B decompensated cirrhosis. We conducted a meta-analysis on virological, serological, biochemical reactions, drug-resistance, mortality and adverse reactions in the included trials to examine the association between pre-specified characteristics (treatment duration) and treatment effect.

HBV DNA level is a primary prognostic marker and risk factor for patients with hepatitis B decompensated cirrhosis<sup>[29]</sup>. The early and sustained suppression of HBV DNA replication is associated with improved long-term virological, serological and biochemical response rates. Rapid and effective suppression of HBV DNA replication can reduce the incidence of LC, HCC and drug-resistance<sup>[30,31]</sup>. The results of our meta-analysis showed that following various treatment durations (12, 24, 36, 48 and > 48 wk), HBV DNA levels were lower in the ETV group than in the LAM group, and the difference between the two groups was statistically significant. At 12, 24 and 48 wk of treatment, ETV showed a significant effect on the rate of HBV DNA undetectability compared with LAM. These results showed that ETV was not only more effective than LAM in the early stages of treatment, but also had a continuous advantage after treatment. This suggests that ETV had a more rapid and effective anti-viral activity in patients with hepatitis B decompensated cirrhosis than LAM. Although HBeAg seroconversion in the ETV group was more pronounced than in the LAM group at 24 wk (27.9% *vs* 26.19%) and 48 wk (31.52% *vs* 25%) of treatment, these differences were not statistically significant.

Following various treatment durations, both ETV and LAM significantly reduced ALT, TBIL and CTP levels and increased ALB and PTA levels. These results indicated that both drugs significantly improved liver function.

ETV has a high genetic barrier to resistance<sup>[32]</sup>. The results in Figure 2D show that the rate of drug-resistance was higher in the LAM group (17.12%) than in the ETV group (0.44%), and this difference was statistically significant. ETV has lower drug-resistance, and is thus more suitable for the treatment of patients with hepatitis B decompensated cirrhosis than LAM.

The results in Figure 2G show that the rate of mortality in the two treatment groups was similar (ETV 6.37% *vs* LAM 7.89%), and no statistically significant difference was observed. No severe adverse reactions were observed in the two treatment groups. These results suggest that both ETV and LAM significantly reduced mortality, with excellent safety and tolerability.

Our study had several limitations. First, the number of included trials was small, and some outcome param-

eters of treatment duration included only 1 trial. Second, the quality of some of the included trials was not high (details on the method of randomization, allocation concealment, blinding method, or the reasons for withdrawals and dropouts were not included). Therefore, future studies should assess high-quality, well-designed, multicenter RCTs with larger sample sizes.

In conclusion, both ETV and LAM have powerful anti-viral activity, with a low incidence of adverse reactions. These drugs also improved liver function and reduced mortality. Therefore, the positive effects of ETV and LAM in patients with hepatitis B decompensated cirrhosis were confirmed. Due to lower cost, LAM has a pharmacoeconomic advantage before 48 wk of treatment. However, LAM has higher drug-resistance, and is thus unsuitable for the long-term treatment of patients with hepatitis B decompensated cirrhosis. ETV can be used as the first-line drug for long-term treatment of patients with hepatitis B decompensated cirrhosis due to its greater anti-viral activity and extremely low drug-resistance.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The treatment of chronic hepatitis B is a major healthcare problem affecting over 350 million people worldwide. Approximately 25%-40% of infected patients will develop various life-threatening conditions such as liver failure, liver cirrhosis and hepatocellular carcinoma. Recent studies have shown that entecavir (ETV) and lamivudine (LAM) are powerful nucleoside analogues in the treatment of hepatitis B decompensated cirrhosis. However, there were few systematic reviews on this topic.

### Research frontiers

Lamivudine effectively suppressed hepatitis B virus (HBV) DNA replication and significantly improved liver function in patients with hepatitis B decompensated cirrhosis. However, a critical weak point of lamivudine therapy is the frequent occurrence of resistant mutations and high drug-resistance in HBV. ETV is a new cyclopentyl guanosine nucleoside analogue. It has the advantage of a higher rate of HBV DNA suppression, low drug-resistance and high safety. However, it is expensive and long-term use of ETV would result in heavy financial pressures for patients with hepatitis B decompensated cirrhosis and their families.

### Innovations and breakthroughs

There are few systematic reviews on the efficacy of ETV and LAM in the treatment of hepatitis B decompensated cirrhosis. The authors conducted a meta-analysis of randomized controlled trials using the Cochrane methodology and explored the efficacy of ETV and LAM for the treatment of hepatitis B decompensated cirrhosis.

### Applications

Due to its lower cost, LAM has a pharmacoeconomic advantage before 48 wk of treatment. However, LAM has higher drug-resistance, and is thus unsuitable for the long-term treatment of patients with hepatitis B decompensated cirrhosis. ETV can be used as the first-line drug for the long-term treatment of patients with hepatitis B decompensated cirrhosis due to its greater anti-viral activity and extremely low drug-resistance.

### Terminology

HBV DNA undetectability: undetectable levels of HBV DNA (HBV DNA levels < 1000 copies/mL), determined by quantitative polymerase chain reaction. Hepatitis B e antigen (HBeAg) seroconversion: HBeAg loss (HBeAg levels < 1.0 S/CO)

and the presence of anti-HBeAg, determined by microparticle enzyme immunoassay or enzyme-linked immunosorbent assay. CTP score: employs five clinical measures of liver disease [total bilirubin, albumin, Prothrombin time, Ascites, Hepatic encephalopathy]. Each measure is scored 1-3, class A to C by total scores (A:5-6, B:7-9, C:10-15), with C indicating the most severe liver disease.

### Peer review

This is a good meta-analysis comparing the ETV and LAM in treatment of hepatitis B associated decompensated cirrhosis. Based on their analyses, the authors conclude that both ETV and LAM can significantly improve the liver function and reduce mortality for patients with hepatitis B decompensated cirrhosis. However, ETV has a better virological response and lower drug-resistance, which can be used as the first-line drug for long-term treatment of hepatitis B decompensated cirrhosis. The analysis was carefully performed, and the results were clearly presented and summarized.

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## Aberrant celio-mesenteric supply of the splenic flexure: Provoking a bleed

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### Abstract

Lower gastrointestinal hemorrhage presents a common indication for hospitalization and account for over 300000 admissions per year in the United States. Multimodality imaging is often required to aid in localization of the hemorrhage prior to therapeutic intervention if endoscopic treatment fails. Imaging includes computer tomography angiography, red blood cell tagged scintigraphy and conventional angiography, with scintigraphy being the most sensitive followed by computer tomography angiography. Aberrant celio-mesenteric supply occurs in 2% of the population; however failure to identify this may result in failed endovascular therapy. Computer tomography angiography is sensitive for arterial hemorrhage and delineates the anatomy, allowing the treating physician to plan an endovascular approach. If at the time of conventional angiography, the active bleed is not visualized, but the site of bleeding has been identified on computer tomography angiography, provocative angiography can be utilized in

order to stimulate bleeding and subsequent targeted treatment. We describe a case of lower gastrointestinal hemorrhage at the splenic flexure supplied by a celio-mesenteric branch in a patient and provocative angiography with tissue plasminogen activator utilized at the time of treatment to illicit the site of hemorrhage and subsequent treatment.

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**Key words:** Hemorrhage; Mesenteric arteries; Colon transverse; Thrombolytic therapy; Embolization therapeutic; Computer tomography

**Core tip:** In this article, the authors describe a case of lower gastrointestinal hemorrhage at the splenic flexure supplied by a celio-mesenteric branch in a patient and provocative angiography with tissue plasminogen activator utilized at the time of treatment to illicit the site of hemorrhage and subsequent treatment.

Wu M, Klass D, Strovski E, Salh B, Liu D. Aberrant celio-mesenteric supply of the splenic flexure: Provoking a bleed. *World J Gastroenterol* 2013; 19(39): 6679-6682 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6679.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6679>

### INTRODUCTION

Lower gastrointestinal (LGI) hemorrhages present a common indication for hospitalization and account for over 300000 admissions per year in the United States<sup>[1]</sup>. In the majority of these events, modern imaging and endoscopic techniques such as upper and/or lower endoscopy, tagged red blood cell scintigraphy, and visceral angiography can be used to localize the source of an acute hemorrhage<sup>[2]</sup>. Predictors on the ability to find a bleeding source include: (1) being visible on multidetec-

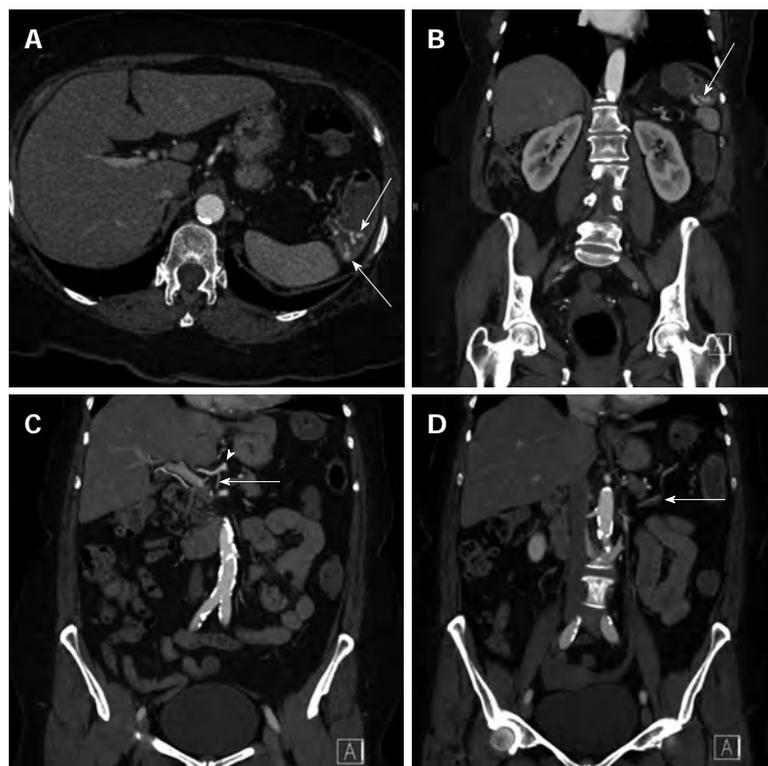


Figure 1 Computed tomography angiography with arterial phase (20 s following injection) acquisition. A, B: Axial image (A) and coronal images (B) demonstrating active contrast extravasation (arrows) at the splenic flexure; C: Coronal reformatted image demonstrating a celio-mesenteric branch (arrow) arising from the common hepatic artery (arrowhead); D: Seen in the large bowel mesentery (arrow) and supplying the splenic flexure.

tor computed tomography angiography (CTA); (2) visible on tagged red blood cell scintigraphy; or (3) patient hemodynamic instability. Yet, in as many as 65% of cases, standard diagnostic evaluation will not identify the source of a bleed and these patients may present with recurrent bleeds from an obscure origin<sup>[3]</sup>. Modern radiologic studies require an active bleed and a minimal rate of bleeding in order to be detected. Since LGI hemorrhages can frequently resolve before imaging is performed, these studies may have difficulty in finding a source of the bleed<sup>[4]</sup>. If the bleeding vessel is not visualized angiographically, a provocative maneuver can be performed using heparin or tissue plasminogen activator (tPA) at the suspected site. This technique is based on the premise that these pharmacologic agents will incite an acute hemorrhage that can then be viewed angiographically. Once visualized, the source may be treated appropriately by embolization to occlude the vessel or by providing visual cues to assist a surgical procedure<sup>[5]</sup>.

In this letter, we discuss the utility of a multidetector CTA scan in the setting of a LGI bleed that revealed the presence of an aberrant vessel supplying the splenic flexure responsible for the hemorrhage. With this information, we describe the use of provocative maneuvers to localize and successfully embolize the source of the bleed.

## CASE REPORT

A 65-year-old female presented with the passage of

bright red blood per rectum, the patient was not hemodynamically unstable (Blood pressure 110/60, pulse 78, saturation on room air 98%). Endoscopy was felt likely to be unhelpful due to the amount of blood passed per rectum and therefore decision was taken to proceed to CT. A subsequent arterial phase CT scan demonstrated an acute hemorrhage at the site of the splenic flexure (Figure 1A and B). Furthermore, a single aberrant vessel was seen arising from the proximal common hepatic artery (CHA) to supply the splenic flexure (Figure 1C and D). The patient was hemodynamically stable and transferred to the angiography suite for therapy.

A celiac angiogram was initiated through the right common femoral artery using a 5-Fr sheath. Access was obtained *via* a Sim 1 catheter (Cook Medical, Bloomington, IN) that extended to the celiac axis. As previously established on CTA, an aberrant celio-mesenteric branch extending from the CHA to the splenic flexure was confirmed. The Sim 1 catheter was engaged further and a Renegade STC catheter (Boston Scientific, Natick, MA) was used to cannulate the vessel supplying the splenic flexure (Figure 2A and B).

Serial angiograms were performed in this area but no acute hemorrhage was visualized (Figure 2B). Decision was made to perform a tPA provocation test. Two milligram of tPA was dissolved in 10ccs of saline. One milligram of tPA was injected selectively through the catheter into the vessel supplying the splenic flexure. This resulted in brisk bleeding in the area of the splenic flexure from the aberrant vessel of the CHA (Figure 2C). At

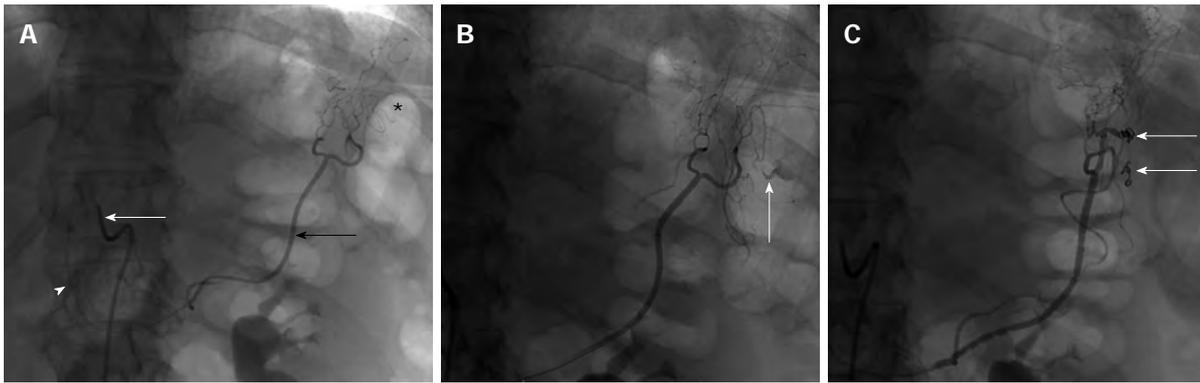


Figure 2 Digital subtraction angiogram. A: Sim 1 catheter (white arrow) engaged in the common hepatic artery and a microcatheter (arrowhead) cannulating the celiac-mesenteric trunk (black arrow) supplying the splenic flexure (asterisk) with no active hemorrhage seen; B: Active contrast extravasation (arrow) following instillation of 1 mg tPA; C: Angiogram following deployment of coils in branches of the celiac-mesenteric vessel and no further active extravasation seen (arrow).

this point, the catheter was advanced and three 3 mm × 3.3 mm Vortex coils were injected in order to close the proximal and distal sites of the bleed. Further selective angiography did not demonstrate any acute contrast extravasation (Figure 2D).

The patient remained hemodynamically stable during the procedure and the blood pressure normalized at approximately 150/70 mmHg following embolization.

## DISCUSSION

Patients with a gastrointestinal bleed of unknown origin present as a challenging population since typical investigations such as CT imaging, red blood cell scintigraphy and endoscopic procedures are unable to find a source to the bleed. Without a known source, these patients are usually subjected to increased risks from repeat bleeding events, invasive investigations, and blood transfusions<sup>[6]</sup>. Pharmacologic agents can be used to incite an acute, local bleed in order to visualize the source of the hemorrhage on CT scan. Although provocative angiography has yet to be a common diagnostic tool, the literature available through case reports and series has shown this to be an effective yet safe technique<sup>[2,4-8]</sup>.

In this particular case, the inclusion of a CT angiogram in the setting of a GI bleed was invaluable in locating the source of the hemorrhage as the patient demonstrated aberrant vasculature from the CHA to the splenic flexure. The presence of variant vessels supplying the descending colon has been well documented and, although uncommon, would be important information in order to guide the management of a bleeding patient<sup>[9-11]</sup>. CTA provides excellent delineation of the arterial anatomy and is more sensitive than conventional angiography in identifying arterial bleeding<sup>[12]</sup>.

The anastomosis between the superior mesenteric artery (SMA) and the inferior mesenteric artery (IMA) at the splenic flexure is normally considered a watershed region with dual arterial supply from the SMA and IMA allowing collateral circulation. This region however is more susceptible to damage in ischemic disease.

In relatively rare cases, this point in the large bowel may receive blood supply from a celiac-mesenteric branch. The anastomosis of atypical coeliac branches represents a rare case for consideration. Awareness of the possibility of embryological variants will assist in minimizing the risk of complications in angiographic procedures. Failure to identify this branch supplying the splenic flexure may lead to an incorrect assessment of the mesenteric vasculature, particularly at the time of angiography. It also provides comprehensive detail of the arterial anatomy and allows the radiologist to assess both access and potential target vessels for treatment.

We propose that in patients with a GI bleed that is difficult to locate, an arterial phase CT scan is a tool that can provide valuable information such as the ability to reveal variant vasculature that would have otherwise gone unnoticed. The protocol does not require oral contrast. Furthermore, if one is confident that there is a bleed but is unable to view angiographically, then a challenge with tPa or heparin may be a useful adjunctive diagnostic approach.

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E- Editor Ma S



## A rare case of primary choriocarcinoma in the sigmoid colon

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### Abstract

Primary colorectal choriocarcinoma is an extremely rare neoplasm and is usually associated with a poor prognosis. Only 13 cases of colorectal choriocarcinoma have previously been reported. There is no standard chemotherapeutic regimen for this tumor type. A 68-year-old man presented with melena and was diagnosed with sigmoid colonic adenocarcinoma with multiple liver metastases. He underwent a laparoscopic sigmoidectomy. Pathology revealed choriocarcinoma with a focal component of moderately differentiated adenocarcinoma of colon origin. Based on the collagen gel droplet-embedded culture drug sensitivity test (CD-DST) results, mFOLFOX6 and bevacizumab were administered, which suppressed aggressive tumor growth for 4 mo. The patient died 9 mo after the initial diagnosis. Our study results suggest that the standard chemotherapy regimen for colorectal cancer might have suppressive effects against primary colorectal choriocarcinoma. Moreover, CD-DST may provide, at least in part, therapeutic insight for the selection of appropriate antitumor agents

for such patients.

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**Key words:** Colon cancer; Colorectal cancer; Chemotherapy; Culture drug sensitivity test; Choriocarcinoma

**Core tip:** Primary colorectal choriocarcinoma is an extremely rare neoplasm and is usually associated with a poor prognosis. Only 13 cases of colorectal choriocarcinoma have previously been reported. Systemic chemotherapy is an important prognostic factor in these patients; however, there is no standard chemotherapeutic regimen for this tumor type. We encountered an extremely rare case of choriocarcinoma of the colon which was treated with mFOLFOX6 and bevacizumab based on the results of the culture drug sensitivity test (CD-DST), which suppressed aggressive tumor growth. This suggests that CD-DST may provide, at least in part, therapeutic insight for selecting appropriate antitumor agents for patients with colorectal choriocarcinoma.

Maehira H, Shimizu T, Sonoda H, Mekata E, Yamaguchi T, Miyake T, Ishida M, Tani T. A rare case of primary choriocarcinoma in the sigmoid colon. *World J Gastroenterol* 2013; 19(39): 6683-6688 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6683.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6683>

### INTRODUCTION

Choriocarcinoma is an uncommon malignant tumor that originates from the placenta in women and from germ cells of the gonads in men. Choriocarcinoma is more common in men than in women. Non-gestational or extra-gonadal choriocarcinoma are rare tumors that arise at various sites including the mediastinum<sup>[1]</sup>, lung<sup>[2]</sup>, stomach<sup>[3]</sup>, pancreas<sup>[4]</sup>, cervix<sup>[5]</sup>, and ureter<sup>[6]</sup>. Primary

choriocarcinoma of the colon and rectum is an extremely rare neoplasm and is usually associated with a poor prognosis. Only 13 cases of colorectal choriocarcinoma have previously been reported<sup>[7-19]</sup>. According to these reports, systemic chemotherapy also appeared to be an important prognostic factor in these patients; however, no standard chemotherapy regimen exists to treat this tumor.

The collagen gel droplet-embedded culture drug sensitivity test (CD-DST) is a new *in vitro* anticancer drug sensitivity test<sup>[20]</sup> that has been reported to provide valuable therapeutic information for patients with colorectal cancer<sup>[21-23]</sup> and pancreatic tumors<sup>[24]</sup>. We encountered an extremely rare case of primary choriocarcinoma of the sigmoid colon. CD-DST results revealed that the resected tumor was sensitive to several antitumor drugs including oxaliplatin (OHP). Based on the CD-DST results, we administered effective chemotherapy to this patient. To the best of our knowledge, this is the first report of the clinical utility of CD-DST in the treatment of a patient with colonic choriocarcinoma.

## CASE REPORT

A 68-year-old man presented with melena. Colonoscopy revealed an ulcerated tumor that occupied half the diameter of the lumen of the sigmoid colon (Figure 1A). A barium enema revealed the tumor to be approximately 2.5 cm in diameter (Figure 1B). Abdominal computed tomography (CT) revealed multiple hepatic tumors and thickening of the intestinal wall of the sigmoid colon. Biopsy of the tumor in the sigmoid colon suggested moderately to poorly differentiated adenocarcinoma. The alpha-fetoprotein (AFP) level was markedly elevated at 298.7 ng/mL (normal range < 20 ng/mL); however, carcinoembryonic antigen (CEA) and CA19-9 levels were within the normal ranges. The patient underwent laparoscopic sigmoidectomy with lymph node dissection to determine the chemosensitivity of the tumor using CD-DST, after which final clinical staging was determined to be T2, N1a, M1a (Stage IVA; Tumor-node-metastasis Classification of Malignant Tumors, 7<sup>th</sup> Ed.). The surgical specimen was identified as a 20 mm × 20 mm tumor, and was microscopically diagnosed as choriocarcinoma with a focal component of moderately differentiated adenocarcinoma. The adenocarcinoma component was located at the bottom of the ulcer (Figure 2). Regional lymph node metastasis on the primary feeding artery of the tumor was identified. Immunohistochemical staining results were positive for beta-human chorionic gonadotropin (hCG-beta) expression, a marker of choriocarcinoma. The plasma hCG-beta level was elevated to 1.4 ng/mL (normal range < 0.5 ng/mL) even after surgery. CD-DST results revealed that the tumor was sensitive to various chemotherapeutic agents (Table 1).

The patient received his first session of mFOLFOX6 3 wk after surgery. Because the CD-DST results indicated that the tumor was most sensitive to 5-fluorouracil (FU)/OHP, which simulates the FOLFOX combination,

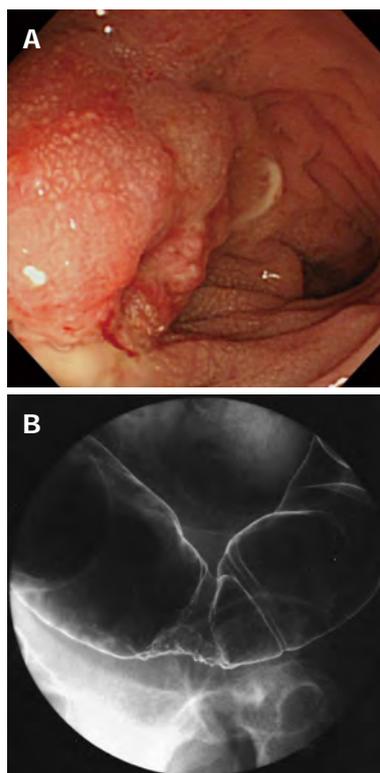
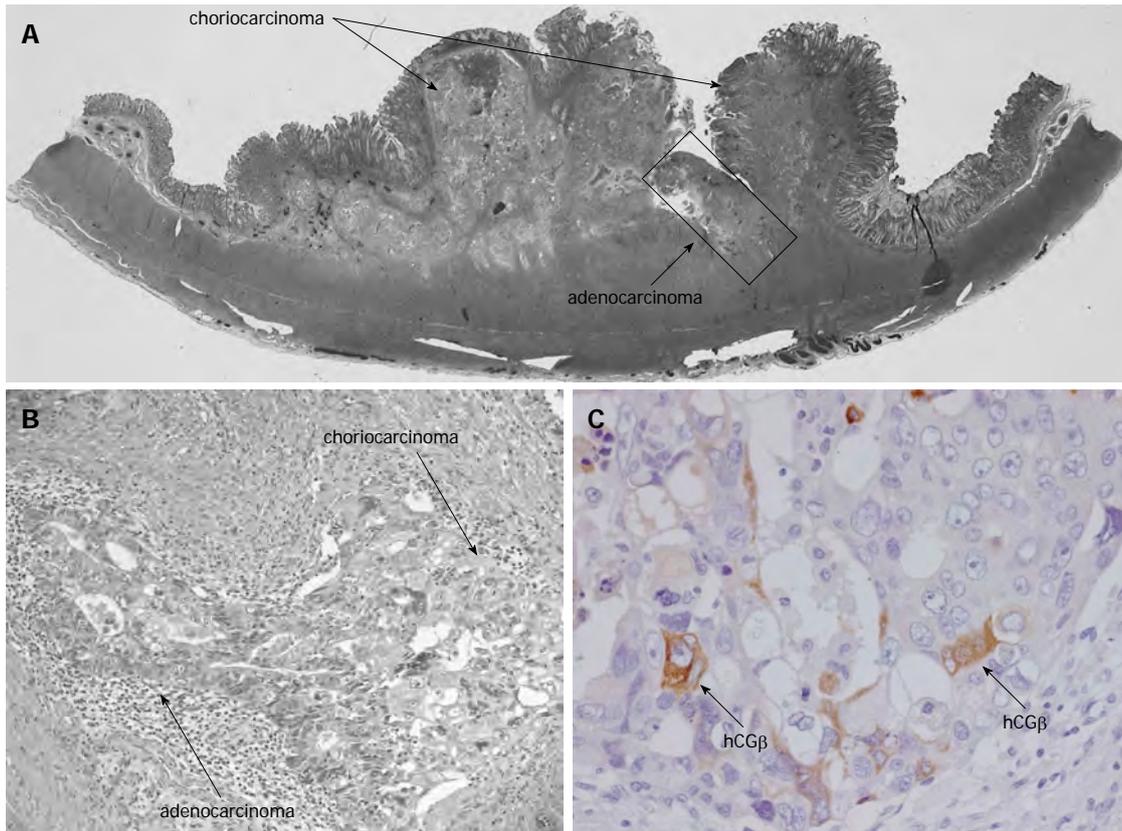


Figure 1 Findings of preoperative examinations. A: Colonoscopy. B: Barium enema.

the patient received a further 8 courses of mFOLFOX6 with bevacizumab. The size of the hepatic metastases remained almost the same as before surgery; however, a small new lesion was observed on abdominal CT 4 mo after surgery. These findings indicated progressive disease according to RECIST criteria. However, at this point we considered the hepatic tumors unlikely to continue growing because of systemic chemotherapy (Figure 3A and B). However, at 6 mo after surgery, serum AFP and hCG-beta levels rapidly elevated to 3929 and 11 ng/mL, respectively. Thereafter, we administered a regimen of FOLFIRI with bevacizumab. Despite this treatment, the hepatic metastases enlarged markedly (Figure 3C), and the patient died 9 mo after the initial diagnosis.

## DISCUSSION

Primary choriocarcinoma of the colon and rectum is extremely rare. To our knowledge only 14 cases including ours have been documented to date. The median age of these patients was 52 years (range, 29-74 years) and included 6 men and 8 women. The median survival period was 4 mo (range, 0.3-60 mo). Metastatic tumors were found in distant organs at the time of colorectal choriocarcinoma diagnosis in 71.4% (10/14) of cases. Of the cases who had no distant metastases at the time of surgery, 80% (4/5) showed rapid development of distant metastases. Only 1 case was reported to have achieved long-term (60 mo), relapse-free survival. These tumors tend to rapidly progress, and are therefore, associated



**Figure 2 Microscopic findings.** A, B: HE staining shows the co-existence of choriocarcinoma and adenocarcinoma cells (A: loupe, B: × 200); C: The tumor cells were positive for β-human chorionic gonadotropin (hCGβ) (× 400).

Table 1 Summary of antitumor drug sensitivity test results	
Agent (μg/mL)	T/C
5-FU (1.0)	53.34%
Gemcitabine (0.03)	58.47%
Docetaxel (0.1)	56.07%
Epirubicin (0.1)	58.47%
Cisplatin (0.2)	UD
5-FU/SN38 (1.0/0.03)	65.85%
5-FU/OHP (1.0/0.5)	46.85%

The culture drug sensitivity test method was employed to study *in vitro* growth inhibition. The *in vitro* sensitivity is expressed as the T/C ratio, where T is the total volume of living cancer cells in the treated group, and C is the total volume of living cancer cells in the control group. 5-FU: 5-fluorouracil; SN38: The active metabolite of irinotecan; OHP: Oxaliplatin; UD: Undetectable because of the limited number of cells.

with very poor prognosis. Almost all of the reported patients [85.7% (12/14)] died within 1 year of diagnosis. The survival rate in patients without chemotherapy was significantly lower than that for patients who received some form of chemotherapy (median survival period, without systemic chemotherapy: 1.0 mo *vs* with systemic chemotherapy: 9 mo, Kaplan-Mayer analysis; Log rank test  $P = 0.0004$ , data not shown). Although intestinal resection with lymph node dissection was the standard treatment for colorectal choriocarcinoma in the previous reports, systemic chemotherapy also appeared to be

an important prognostic factor. However, no standard chemotherapy regimen has been established for the treatment of this tumor type.

Since colorectal choriocarcinoma is generally considered biologically similar to choriocarcinoma of gestational trophoblastic neoplasia (GTN), almost all previously used chemotherapeutic regimens were based on those designed to treat GTN<sup>[25]</sup>. Choriocarcinoma is the most common malignant form of GTN. The standard chemotherapeutic regimen for GTN is EMA/CO (a combination of etoposide, methotrexate, and dactinomycin, alternating with cyclophosphamide and vincristine). EMA/CO is reported to have a 60.0%-90.6% complete remission rate and an 86.2% 5-year overall survival (OS)<sup>[26]</sup>. However, there is no evidence that EMA/CO is effective against colorectal choriocarcinoma.

The prognosis of choriocarcinoma that is of colorectal origin is extremely poor, as shown in this case report. The response of colorectal choriocarcinoma to chemotherapy is much worse than that of choriocarcinoma derived from germ cells. The cause of this difference in chemosensitivity is still unknown. It is known that colorectal choriocarcinoma cells undergo a syncytiotrophoblastic differentiation through retrodifferentiation or metaplasia of the adenocarcinoma component, rather than originating directly from ectopic germ cells. Therefore, it is possible that the differences in chemosensitivity

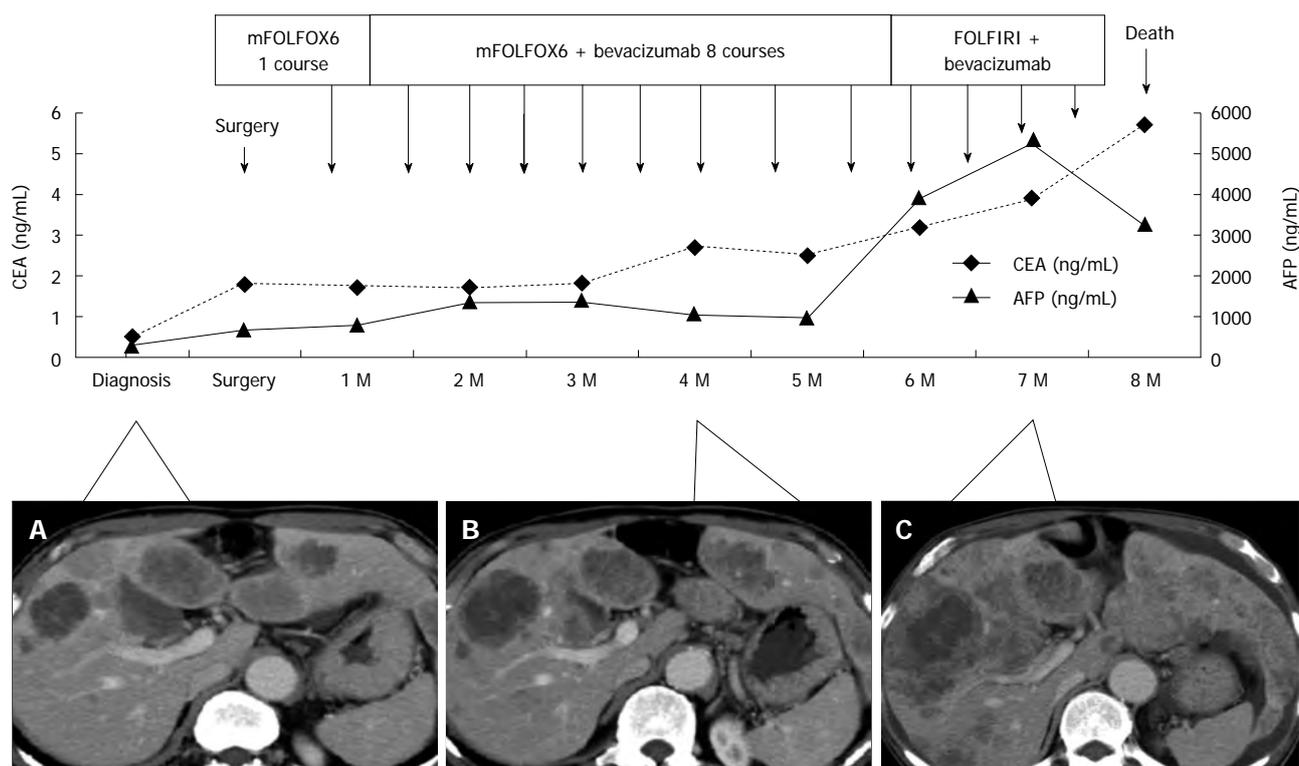


Figure 3 Clinical course of the patient. A, B: Hepatic metastases showed minimal growth until 4 mo after surgery; at 7 mo after surgery; C: The metastases had markedly enlarged, CEA: Carcinoembryonic antigen; AFP: Alpha-fetoprotein.

may be associated with the origin of carcinoma cells.

A study by Harada *et al*<sup>[7]</sup> reported the efficacy of a systemic chemotherapeutic regimen that contained MEA-methotrexate, etoposide, and dactinomycin which targeted the choriocarcinoma, followed by oral administration of tegafur and uracil/leucovorin therapy which targeted the colonic adenocarcinoma. Although this was a report of a single case, the treatment resulted in very long-term, relapse-free survival (60 mo). Therefore, chemotherapy which not only targeted the choriocarcinoma, but also the adenocarcinoma of the large intestine, appeared to be the key to prolonging survival.

Recently, FOLFOX and FOLFIRI have become standard chemotherapeutic regimens for the treatment of colorectal cancer<sup>[27-29]</sup>. However, the use of irinotecan- or OHP-containing regimens to treat colorectal choriocarcinoma has not been reported. In this case, the CD-DST results indicated that the tumor was most sensitive to 5-FU/OHP, which simulates the FOLFOX combination. The tumor sample used in the CD-DST was mainly choriocarcinoma; however, the pathological findings revealed the co-existence of moderate-to-poorly differentiated adenocarcinoma. Noguchi *et al*<sup>[30]</sup> reported that the survival of a patient with gastric choriocarcinoma was prolonged by administering a chemotherapy regimen commonly given to patients with gastric adenocarcinoma, rather than to those with choriocarcinoma. The regimen for adenocarcinoma in the original tissue appeared to be effective against non-gestational or extra-gonadal chorio-

carcinoma. Therefore, we hypothesized that the standard regimen for the treatment of colorectal cancer might be effective against primary colorectal choriocarcinoma. We selected mFOLFOX6 combined with bevacizumab with the approval of the patient.

CD-DST cultures extract cancer cells three-dimensionally in a collagen gel droplet. This three-dimensional culture with collagen matrix is preferable to establish cell cultures from human cancer tissue<sup>[31]</sup>. Here, we report that CD-DST may provide useful information to tailor chemotherapy regimens to individual patients<sup>[21,23,24]</sup>. Our study demonstrates that patients with synchronous stage IV colorectal cancer who were treated with tumor-sensitive chemotherapeutics as evidenced by the CD-DST had higher response rates (85.71%) than patients receiving drugs that the CD-DST did not identify as tumor-sensitive (41.67%). Moreover, progression-free survival (PFS) and OS were superior in patients treated with *in vitro* sensitive drugs by CD-DST (median PFS, 696.5 d *vs* 297.5 d; median OS, 1023.4 d *vs* 518.5 d)<sup>[23]</sup>. Unfortunately, the cell culture was not maintained long enough for additional examination of anti-tumor drugs for the EMA/CO regimen after the results of immunohistochemical staining were obtained. Although the best regimen according to the results of the CD-DST suppressed rapid progression of choriocarcinoma in this case, the therapeutic effect in this patient was not remarkable compared with our previous findings of the CD-DST in common colorectal adenocarcinoma<sup>[23]</sup>. We could not evaluate hepatic meta-

static lesions using the CD-DST in this case. It is possible that the chemosensitivity of the hepatic metastases may be different from that of the primary lesion.

In conclusion, we report an extremely rare case of primary choriocarcinoma of the sigmoid colon. Systemic chemotherapy appeared to improve the patient's survival. This case suggests that the standard regimen for the treatment of colorectal cancer might have suppressive effects against primary colorectal choriocarcinoma. Moreover, CD-DST may provide, at least in part, therapeutic insight for the selection of appropriate antitumor agents which may be effective for treating patients with colonic choriocarcinoma on an individual basis.

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## Diagnosis and treatment of benign multicystic peritoneal mesothelioma

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### Abstract

Benign multicystic peritoneal mesothelioma (BMPM) is a rare cystic mesothelial lesion that occurs predominantly in reproductive aged women. A 56-year-old Caucasian male was admitted to our surgical department with a chief complaint of a painful mass in his right lower abdomen for almost 2 years. The physical examination revealed a palpable painful mass. Computed tomography demonstrated an irregular, cystic tumor in his right lower abdomen. There was no obvious capsule or internal septations. No enhancement after intravenous administration of contrast was noted. An exploratory laparotomy was performed, and a multicystic tumor and adherent to the caecum was noted. The walls of the cysts were thin and smooth, filled with clear fluid, and very friable. An *en bloc* resection of the tumor, including appendix and caecum, was performed. Histological examination revealed multiple cysts lined with flattened simple epithelial cells, and the capsule walls of

the cysts were composed of fibrous tissue. Immunohistochemical analysis documented positive expression of mesothelial cells and calretinin. The final diagnosis was BMPM. The patient was well at 6-mo follow-up. BMPM is exceedingly rare lesion. A complete resection of the tumor is required. The diagnosis of BMPM is based on pathological analysis.

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**Key words:** Benign multicystic peritoneal mesothelioma; Computed tomography; Pathological analysis; Diagnosis; Resection

**Core tip:** Benign multicystic peritoneal mesothelioma (BMPM) is a rare cystic mesothelial lesion that occurs predominantly in reproductive aged women. The preoperative diagnosis of BMPM is difficult, and final diagnosis requires histological evaluation of a surgical specimen. In immunohistochemical analysis, positive expression of mesothelial cells and calretinin is always noted. The best treatment strategy for BMPM is *en bloc* removal, which can avoid recurrence. The recurrent rate after complete resection is about 50%; the recurrent tumor should be completely removed and follow-up including physical examination and imaging studies is required for all cases.

Wang TB, Dai WG, Liu DW, Shi HP, Dong WG. Diagnosis and treatment of benign multicystic peritoneal mesothelioma. *World J Gastroenterol* 2013; 19(39): 6689-6692 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6689.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6689>

### INTRODUCTION

Benign multicystic peritoneal mesothelioma (BMPM) is uncommon lesion usually occurring in women of repro-

ductive age. Because of its rarity, preoperative diagnosis is difficult and its origin and pathogenesis are uncertain<sup>[1-3]</sup>. Here, we present a case of BMPM in a 56-year-old Caucasian male with a painful mass in right lower abdomen for 2 years. The preoperative diagnosis was angiolymphoma. An *en bloc* removal was performed. The final diagnosis was BMPM according to the findings of histological and immunohistochemical examinations. We hope that this information assists surgeons in recognizing the diagnosis and treatment of BMPM.

## CASE REPORT

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A 56-year-old Caucasian male was admitted to our surgical department with a chief complaint of a painful mass in his right lower abdomen for almost 2 years. His medical history was negative, and he smoked 20 cigarettes per day and stated mild alcohol consumption for 30 years. His vital signs were normal, and physical examination revealed a palpable painful mass in his right lower abdomen. Laboratory investigations, urine analysis, chest and abdominal radiographs did not reveal any abnormalities.

Computed tomography (CT) demonstrated a 13 cm × 12.9 cm × 6.1 cm irregular, cystic tumor in his right lower abdomen. There was no obvious capsule or internal septations. The Hounsfield value was 8, which demonstrated its cystic nature. No enhancement after intravenous administration of contrast was noted (Figure 1). Angiolymphoma was considered preoperatively.

An exploratory laparotomy was performed under general anesthesia, and a multicystic tumor occupying the right abdomen and adherent to the caecum was noted. The walls of the cysts were thin and smooth, filled with clear fluid, and very friable (Figure 2). The multicystic mass, appendix, and caecum were removed. Histological examination revealed multiple cysts lined with flattened simple epithelial cells, and the capsule walls of the cysts were composed of fibrous tissue (Figure 3A). Immunohistochemical analysis documented positive expression of mesothelial cells (MC, Figure 3B) and calretinin (CR, Figure 3C), while expressions of D2-40, CD31, and CD34 were negative (Figure 3D-F). The final diagnosis was BMPM. The patient was discharged in good condition on postoperative day 10, and was free of symptoms at 6-mo follow-up.

## DISCUSSION

Mesotheliomas always arise from pleural, pericardial, and peritoneal lining cells. BMPM is a rare lesion, and until 2009 only about 146 cases were documented in literature<sup>[2]</sup>. The characteristic behavior of BMPM is benign; however, rare cases of malignant transformation have been noted<sup>[4]</sup>.

The pathogenesis of BMPM is still unclear; however, as the majority of cases occur reproductive aged females,

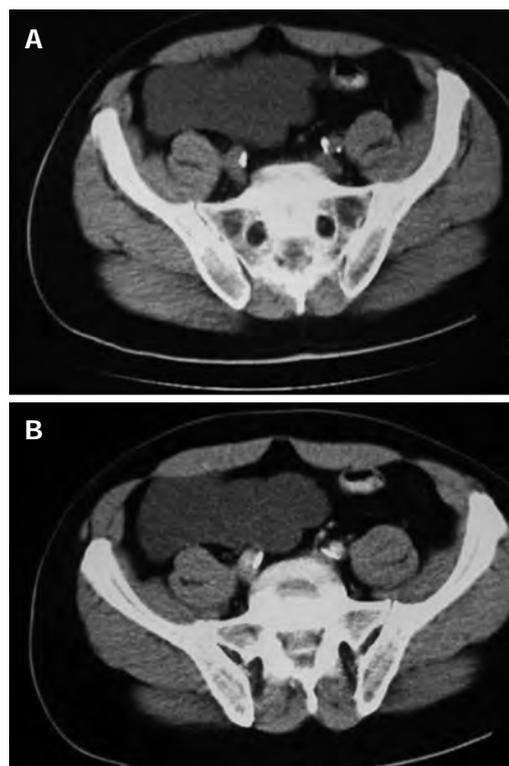


Figure 1 Computed tomography axial image results. A: Plain computed tomography axial image showing a hypodense mass; B: Contrast enhancing computed tomography axial image demonstrating an intra-peritoneal hypodense non-enhancing tumor.

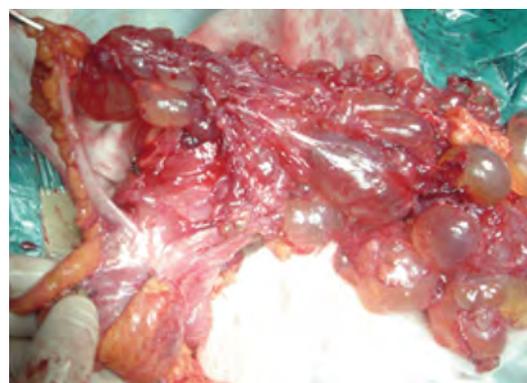
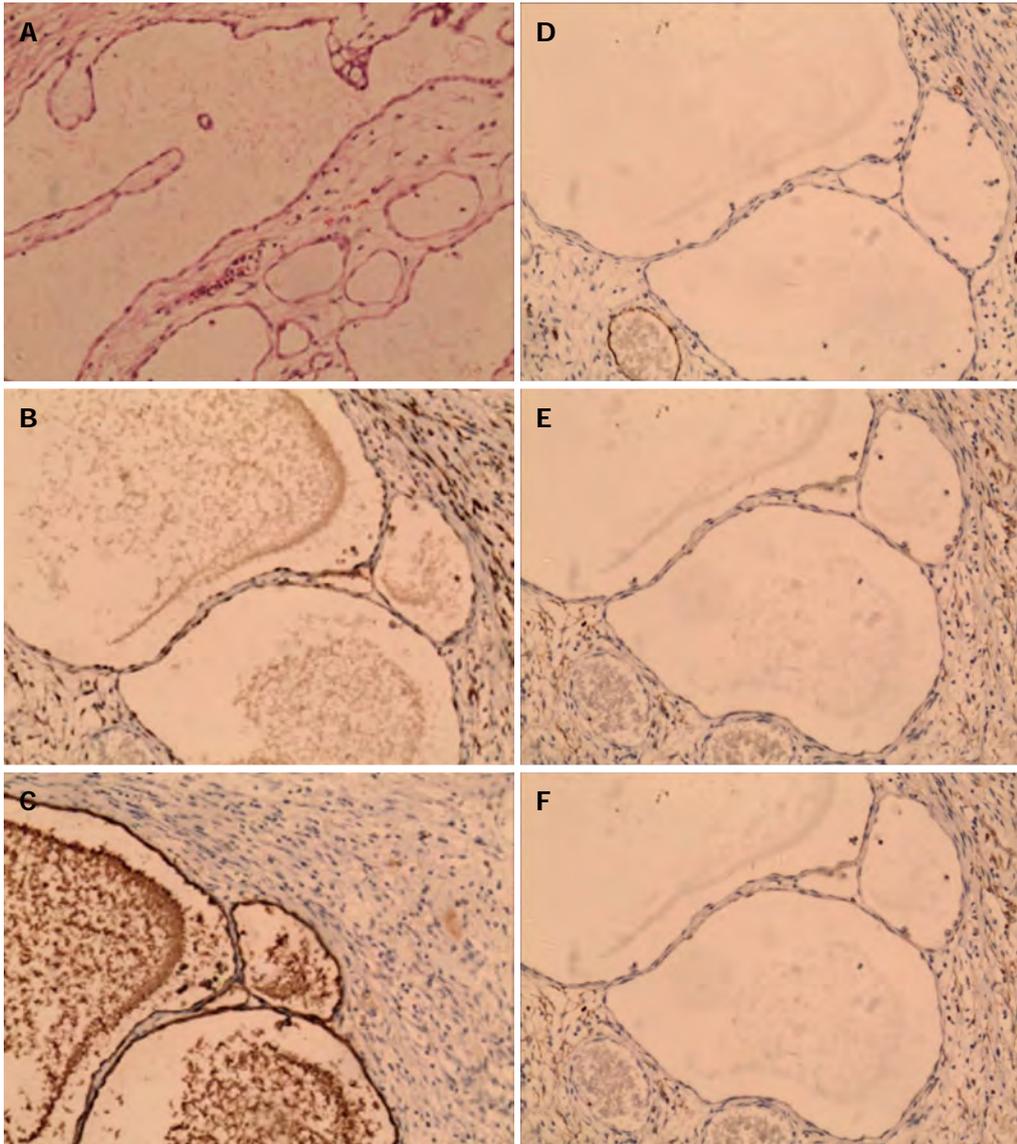


Figure 2 Intraoperative image showing a multicystic mass.

it is believe that female sex hormones play a role in its pathogenesis<sup>[5,6]</sup>. Kurisu *et al*<sup>[7]</sup> reported finding small foci of endometriosis in BMPM cystic walls, and in a second case the lesion was adjacent to endometriotic cysts in the pelvic space. These histologic findings suggest that endometriosis contributes to the origin of BMPM. A history of right oophorectomy and left ovarian cystectomy for an ovarian tumor in a 23-year-old Japanese female with BMPM suggests that previous abdominal surgery is a risk factor for BMPM<sup>[8]</sup>. Husain *et al*<sup>[9]</sup> reported two cases of BMPM in females with concurrent colonic adenocarcinoma arising in the ileocecal region.

The most common presenting symptoms are chronic



**Figure 3** Histological examination revealed results. A: Mesothelial cells lining the cysts ( $\times 400$ ); B-F: Immunohistochemical analysis documented positive expression of mesothelial cells (B,  $\times 400$ ) and calretinin (C,  $\times 400$ ), while expressions of D2-40, CD31, and CD34 were negative (D, F,  $\times 400$ ).

or intermittent abdominal or pelvic pain, tenderness, or distension with an abdominal or pelvic mass. The mean diameter of BMPM has been reported to be 13 cm at the time of diagnosis. On physical examination, the mass always presents as a fixed tumor with slight tenderness. Multiseptated anechoic cysts are always demonstrated by ultrasonography; however, CT is necessary to provide information about the location and extent of the mass and evidence of low-attenuation and non-calcified septa in the cysts<sup>[4]</sup>. Kemp *et al*<sup>[5]</sup> reported a case of BMPM in a middle-aged female that was diagnosed by fine needle core biopsy. An association between BMPM and increased serum CA19-9 concentration has been described, and a minimally invasive laparoscopic approach enabled not only histologic diagnosis but also surgical treatment<sup>[6]</sup>.

Because BMPM is an extremely rare lesion, pre-operative diagnosis is challenging. Definitive diagnosis relies on histological examination, which demonstrates

multiple cysts with thin walls lined with single-layered or cuboidal mesothelia and filled with serous fluid. In immunohistochemical analysis, positive expression of MC and CR, which are markers for mesothelial cells, is always noted. The differential diagnosis primarily includes lymphangioma and malignant peritoneal mesothelioma (MPM). Lymphangioma can be identified if cysts present with predominantly chylous fluid, lymphoid aggregates, smooth muscle, and CD2-40 positive expression on immunohistochemical analysis. MPM always presents a history of exposure to asbestos, abdominal pain, distension, ascites, and weight loss. A plain chest radiograph may show signs of asbestos. An abdominal CT examination may show the presence of ascitic fluid and peritoneal thickening. At laparotomy, widespread nodular thickening of the visceral peritoneum with a striking, diffusely uniform, erythematous appearance can be confirmed to be MPM<sup>[10]</sup>.

The best treatment strategy for BMPM is *en bloc* removal, which can avoid recurrence. The correlation between rupture of cystic lesion of BMPM and recurrence tumor is uncertain. Because of its benign nature, adjuvant chemotherapy and/or radiotherapy are not indicated for patients with BMPM. Other treatments such as hormonal therapy, sclerotherapy, and thermotherapy have not been proven to provided uncertain therapeutic effects. Although the prognosis is excellent, the recurrent rate after complete resection is about 50%; thus, the goal should not be a cure, but symptomatic relief through individualization of treatment. For the rare cases in which malignant transformation occurs, the recurrent tumor should be completely removed and follow-up including physical examination and imaging studies is required for all cases<sup>[11-13]</sup>.

BMPM is a rare tumor, which is not associated with specific complaints, objective signs, or unique appearance on imaging studies. Preoperative diagnosis is very difficult, and the final diagnosis always requires pathological analysis. *En bloc* removal of BMPM is the ideal treatment strategy, and malignant transformation is rare. Further studies are needed to better understand its etiology and pathogenesis.

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## Laparoscopic transduodenal local resection of periampullary neuroendocrine tumor: A case report

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resection; Neuroendocrine tumor; Periampullary tumor

**Core tip:** There are few studies on laparoscopic transduodenal local resection. Only three cases have been reported in the English-language literature. We present our experience in laparoscopic transduodenal local resection in a case of periampullary neuroendocrine tumor. The successful outcome suggests that laparoscopic transduodenal local resection is a feasible procedure in selected patients with periampullary tumor.

Zhang RC, Xu XW, Wu D, Zhou YC, Ajoodhea H, Chen K, Mou YP. Laparoscopic transduodenal local resection of periampullary neuroendocrine tumor: A case report. *World J Gastroenterol* 2013; 19(39): 6693-6698 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6693.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6693>

### Abstract

Studies on laparoscopic transduodenal local resection have not been readily available. Only three cases have been reported in the English-language literature. We describe herein a case of 25-year-old woman with periampullary neuroendocrine tumor (NET). Endoscopic ultrasonography revealed a duodenal papilla mass originated from the submucosa and close to the ampulla. The periampullary tumor was successfully managed with laparoscopic transduodenal local resection without any procedure-related complications. Pathological examination showed a NET (Grade 2) with negative margin. The patient was followed up for six months without signs of recurrence. This case suggests that laparoscopic transduodenal local resection is a feasible procedure in selected patients with periampullary tumor.

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**Key words:** Laparoscopic surgery; Transduodenal local

### INTRODUCTION

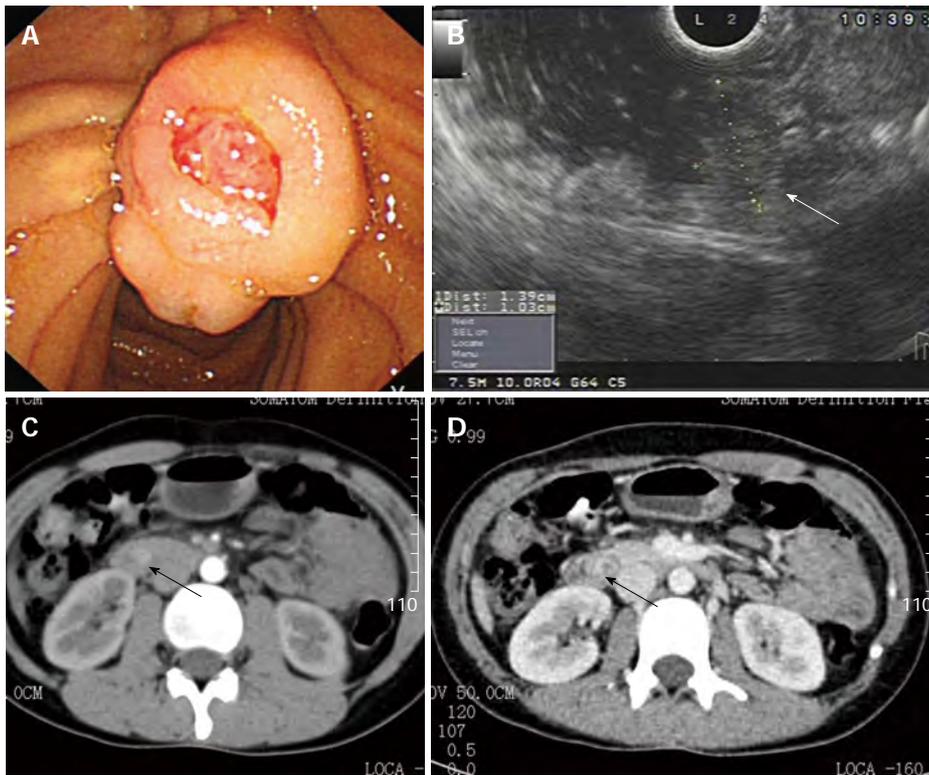
Halsted<sup>[1]</sup> reported the first transduodenal local resection for a patient with adenocarcinoma of the ampulla of Vater. Compared with the traditional Whipple surgery, transduodenal local resection is an organ-preserving operation with low morbidity and mortality<sup>[2]</sup>. Laparoscopic surgery has become widespread because of the improvement of laparoscopic equipment and techniques. However, studies on laparoscopic transduodenal local resection have not been readily available. Only three cases have been reported in the English-language literature (Table 1)<sup>[3,4]</sup>. We herein present our experience in laparoscopic transduodenal local resection in a case of periampullary neuroendocrine tumor (NET).

### CASE REPORT

A 25-year-old woman was admitted to our department because of recurrent melena for 5 mo. She had no fever,

**Table 1** Reported cases of laparoscopic transduodenal local resection

Author	Age (yr)	Gender	Pathology	Size (cm)	Resection margin (cm)	Operative time (min)	Blood loss (mL)	Complication	Postoperative hospital stay (d)
Rosen <i>et al</i> <sup>[3]</sup>	75	Female	Villous adenoma	1.5-2.0	1.0	240	50	-	6
Ahn <i>et al</i> <sup>[4]</sup>	75	Female	Tubular adenoma	2.0 × 1.0 × 0.2	0.5	200	< 50	-	9
Ahn <i>et al</i> <sup>[4]</sup>	55	Male	Gangliocytic paraganglioma	1.0 × 0.9 × 0.7	0.4	250	< 50	-	8



**Figure 1** Periampullary tumor (arrow) was detected by gastroscop, endoscopic ultrasonography, and computed tomography. A: Gastroscop; B: Endoscopic ultrasonography; C, D: Computed tomography.

no abdominal pain, no nausea or vomiting, no diarrhea and no weight loss. She had anemic appearance, and other physical examinations were unremarkable. The laboratory tests showed reduced hemoglobin (7.7 g/dL) and fecal occult blood test was positive. Other laboratory tests, including renal and liver function tests and tumor markers (carcinoembryonic antigen, alpha fetoprotein, and carbohydrate antigens 19-9, 724, 242) were all within normal ranges. Gastroscop showed a duodenal papilla mass (1.2 cm in diameter) with ulcer (Figure 1A). Endoscopic ultrasonography (EUS) revealed a duodenal papilla mass (1.0 cm × 1.4 cm) originated from the submucosa and close to the ampulla (Figure 1B). The pathologic report of the endoscopic biopsy showed small intestinal mucosa, chronic inflammation and focal activity. Computed tomography (CT) disclosed a 1.0 cm × 1.4 cm mass in the descending part of duodenum with rich blood supply (Figure 1C and D). According to the medical history and the imaging findings, the preoperative diagnosis was a periampullary tumor with bleeding [either

NET or gastrointestinal stromal tumor (GIST)]. Laparoscopic transduodenal local resection was performed.

The patient was placed in supine position under general anesthesia. The surgeon and the second assistant who held the laparoscope stood on the right side of the patient and the first assistant stood on the left. Carbon dioxide pneumoperitoneum was established (CO<sub>2</sub> at 15 mmHg) using a Veress needle. One initial 10-mm trocar was placed for the laparoscopy below the umbilicus. A 30-degree telescope was inserted to examine the peritoneal cavity to rule out metastatic disease. After general examination, the other four trocars (one 12 mm and three 5 mm) were inserted into the left upper flank, left flank, right upper flank, and right flank quadrants, respectively; and five trocars were arranged in a V-shape (Figure 2).

Dissection of Calot's triangle was performed carefully. After confirming the cystic artery and cystic duct, the cystic artery was clipped with a 10-mm disposable clip and divided. A small incision of cystic duct was made, and a cholangiogram catheter was inserted through

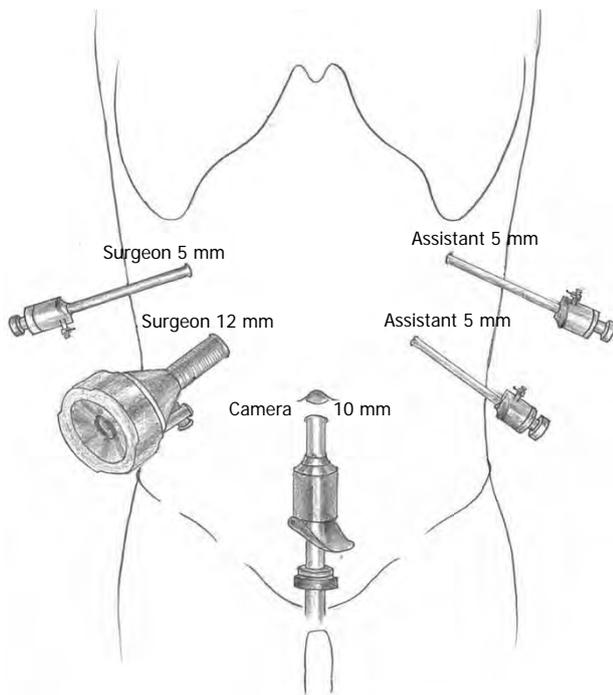


Figure 2 Location of trocars placement.

the cystic duct exiting into the duodenum through the papilla (Figure 3A). The duodenum was mobilized by the Kocher maneuver using harmonic scalpel (Harmonic Ace scalpel, Ethicon Endo-Surgery, Inc., Cincinnati, OH, United States) (Figure 3B). Two stay sutures were placed in the duodenal wall opposite the duodenal papilla, and a longitudinal incision (approximately 3.5 cm) of duodenal wall was made between the stay sutures using harmonic scalpel (Figure 3C). The periampullary tumor was then everted from this duodenotomy by a stay suture without directly manipulating it. The resection was performed circumferentially (inferior to superior) with the harmonic scalpel and electrocautery at a distance of 5 mm from the tumor (Figure 3D). The pancreaticobiliary duct was identified by the cholangiogram catheter passing through it (Figure 3E), and dissected proximally to ensure an adequate margin. Then the specimen was drawn into an endoscopic retrieval bag and removed through the umbilical incision. Intraoperative frozen section confirmed a NET with negative margin. The cut end of pancreaticobiliary duct was visualized within the resected area on the duodenal wall. Under the guidance of cholangiogram catheter, the pancreaticobiliary duct was sutured to the surrounding duodenal mucosa with interrupted 4-0 vicryl suture (Coated polyglactin 910 suture, Ethicon Products, Johnson and Johnson, New Jersey, United States) (Figure 3F). The duodenotomy was closed transversely with an endoscopic linear stapler (Endocutter 60 staple, white cartridge; Ethicon Endo-Surgery, Inc., Cincinnati, OH, United States) and interrupted 3-0 vicryl suture (Figure 3G). The gallbladder was then dissected from the liver bed using harmonic scalpel. The gallbladder was collected in an endoscopic retrieval bag and removed through the

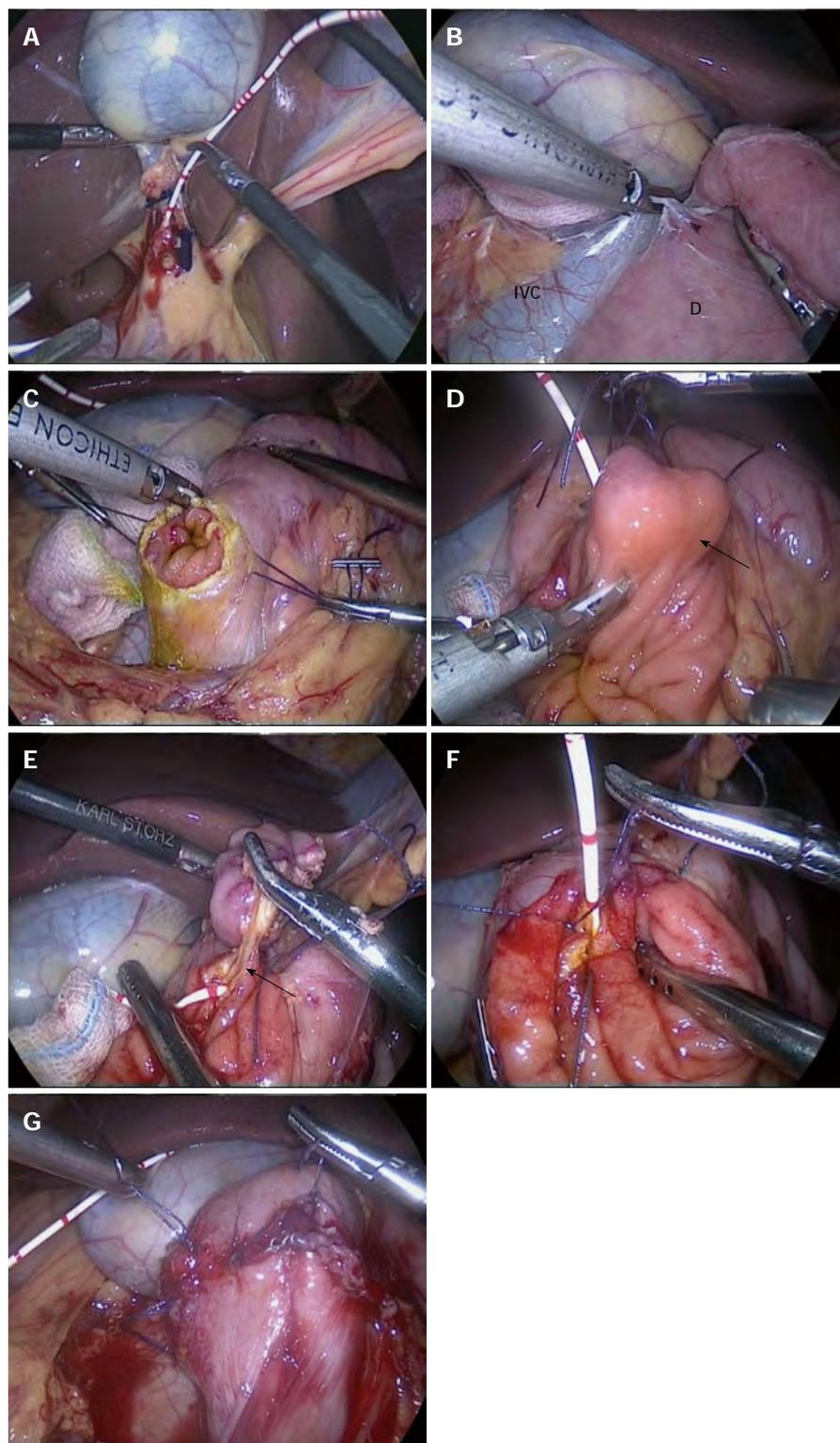
umbilical incision. Two silicon drains were placed adjacent to the duodenum.

The operative time was 180 min and blood loss was 40 mL. The postoperative course was uneventful. The patient started to take semi-fluid on day 6 after surgery, and she was discharged on postoperative day 9. Postoperative pathology showed a NET (Grade 2). The tumor size was 1.3 cm × 0.6 cm × 0.6 cm with negative surgical margin (Figure 4). She was followed up by gastroscopie and CT six months later without signs of recurrence and bleeding.

## DISCUSSION

Duodenal NETs comprises up to 3% of all duodenal tumors and 2%-3% of all endocrine tumors<sup>[5,6]</sup>. Approximately 20% of duodenal NETs occur in the periampullary region<sup>[5]</sup>. Options for resection of small periampullary NETs include pancreaticoduodenectomy (PD), transduodenal local resection, and endoscopic resection<sup>[6]</sup>. Although the mortality rate after PD (less than 4%) has been significantly decreased over recent decades, PD still carries a high morbidity rate ranging from 20% to 60%<sup>[7-9]</sup>. Moreover, pancreatic exocrine insufficiency can affect more than 50% of patients, and diabetes can occur in more than 10% of the patients after PD<sup>[10,11]</sup>. Endoscopic resection is an attractive method for treating benign periampullary tumors<sup>[2]</sup>. But endoscopic resection can only be applied to small tumors without involving the ampulla and pancreatic and biliary ducts<sup>[2]</sup>. Therefore, transduodenal local resection with low morbidity and mortality can be an intermediate treatment option between PD and endoscopic resection in the management of periampullary tumors<sup>[2,12,13]</sup>. In our case, the periampullary tumor had rich blood supply and was close to the ampulla. A probable periampullary NET or GIST was diagnosed before operation. CT and EUS demonstrated the primary tumor, but no sign of locoregional lymph node or distant metastases. Therefore, transduodenal local resection was an optimal choice of treatment for the patient. The final pathological diagnosis was NET (Grade 2), so periodical follow-up after transduodenal local resection for surveillance of recurrence is indispensable.

Because of the complexity of the anatomy of the ampulla and the difficulties in rebuilding the pancreaticobiliary duct system, laparoscopic transduodenal local resection has developed very slowly. Since 2003, when Rosen *et al*<sup>[3]</sup> reported the first case of laparoscopic resection of a periampullary villous adenoma, only three cases using this procedure have been reported in the English-language literature (Table 1)<sup>[3,4]</sup>. The feasibility of this approach is supported by previous cases reported and our successful intraoperative and postoperative results. Compared with the open surgery, the laparoscopic transduodenal local resection is associated with a lower blood loss and perioperative morbidity, and shorter hospital stay, which showed its advantages as a minimally invasive operation (Tables 1 and 2)<sup>[3,4,12-20]</sup>. However, although



**Figure 3** Steps of the surgical procedures. A: Inserting a cholangiogram catheter through the cystic duct to the duodenum; B: Mobilizing duodenum by the Kocher maneuver; C: Making a longitudinal incision of duodenal wall on the opposite site of the duodenal papilla; D: Performing the resection circumferentially at a distance of 5 mm from the tumor (arrow); E: Identifying pancreaticobiliary duct (arrow) by the cholangiogram catheter; F: Suturing the pancreaticobiliary duct to the surrounding duodenal mucosa; G: After closure of the duodenotomy. IVC: Indicates inferior vena cava; D: Duodenum.

laparoscopic transduodenal local resection is a promising procedure, it needs to be validated by more clinical data.

Similar to the open surgery, laparoscopic transduodenal local resection raises two key points: (1) An adequate

margin; and (2) Reconstruction skill for restoration of ductal anatomy. To decrease the likelihood of recurrence, it is important to obtain an adequate margin<sup>[2,12]</sup>. Adequate preoperative evaluation and careful performance

Table 2 Main Published series of open transduodenal local resection

Author	No	Operative time (min)	Blood loss (mL)	Morbidity	Mortality	Reoperation	Length of hospital stay (d)
Park <i>et al</i> <sup>[12]</sup>	4	258.8 <sup>1</sup>	NA	25%	0%	0%	16.5 <sup>1</sup>
Kim <i>et al</i> <sup>[13]</sup>	21	NA	NA	23.8%	0%	0%	9 <sup>2</sup>
Posner <i>et al</i> <sup>[14]</sup>	21	NA	NA	48%	0%	0%	14 <sup>1</sup>
Bohra <i>et al</i> <sup>[15]</sup>	15	NA	NA	13.3%	0%	0%	13 <sup>2</sup>
Sa Cunha <i>et al</i> <sup>[16]</sup>	10	NA	NA	10%	0%	0%	18 <sup>1</sup>
Ouaïssi <i>et al</i> <sup>[17]</sup>	8	NA	NA	25%	0%	0%	15 <sup>1</sup>
Dixon <i>et al</i> <sup>[18]</sup>	19	NA	230 <sup>1</sup>	21.1%	0%	5.35%	NA
Feng <i>et al</i> <sup>[19]</sup>	25	178 <sup>2</sup>	220 <sup>2</sup>	8%	0%	0%	NA
Ceppa <i>et al</i> <sup>[20]</sup>	41	NA	NA	42%	0%	15%	10.1 <sup>1</sup>

<sup>1</sup>Mean; <sup>2</sup>Median. NA: Not available.



Figure 4 Resected specimen of periampullary neuroendocrine tumor (arrow).

of intraoperative frozen section of the margin play a decisive role in ensuring the negative margin<sup>[12]</sup>. Schoenberg *et al*<sup>[21]</sup> performed intraoperative frozen section of the macroscopically normal mucosal tissue 1 cm around the excised lesion in each case with no recurrence after a median 43-mo follow-up. This approach could explain the low recurrence rates in their series. If the margin is involved, conversion to PD should be considered<sup>[12]</sup>. In our case and previous reported cases, intraoperative frozen section of the margins was all performed with negative result. Laparoscopic suturing for restoring ductal anatomy is the most difficult step of the procedure that needs highly skilled suture technique and patience. With the help of high-resolution imaging of the laparoscopy, the surgeon can suture better and ensure each stitch without omission. We inserted a cholangiogram catheter through the cystic duct exiting into the duodenum through the papilla in advance. The catheter was used not only to identify pancreaticobiliary duct during the resection, but also to guide the suturing of pancreaticobiliary duct to the duodenum.

In conclusion, our case suggests that laparoscopic transduodenal local resection is a feasible procedure in selected patients with periampullary tumor. Adequate preoperative evaluation, careful performance of intraoperative frozen section and highly skilled laparoscopic technique are the key factors of success in the laparoscopic transduodenal local resection.

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## Positron emission tomography/computerized tomography in the evaluation of primary non-Hodgkin's lymphoma of prostate

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### Abstract

Primary malignant lymphoma of the prostate is exceedingly rare. Here we report a case of a 65-year-old man who presented with increased urinary frequency, urinary urgency, and urinary incontinence for two years. Benign prostatic hypertrophy was suspected at primary impression. Ultrasound revealed a hypoechoic lesion of the prostate. The total serum prostate-specific antigen was within normal range. Positron emission tomography/computerized tomography (PET/CT) showed a hypermetabolic prostatic lesion. Prostate biopsy was consistent with a non-germinal center diffuse large B cell lymphoma. There was complete remission of the prostatic lesion following six cycles of chemotherapy as shown on the second PET/CT imaging.  $^{18}\text{F}$ -fluoro-deoxy glucose

PET/CT is not only a complement to conventional imaging, but also plays a significant role in the diagnosis and evaluation of treatment response of prostatic lymphoma.

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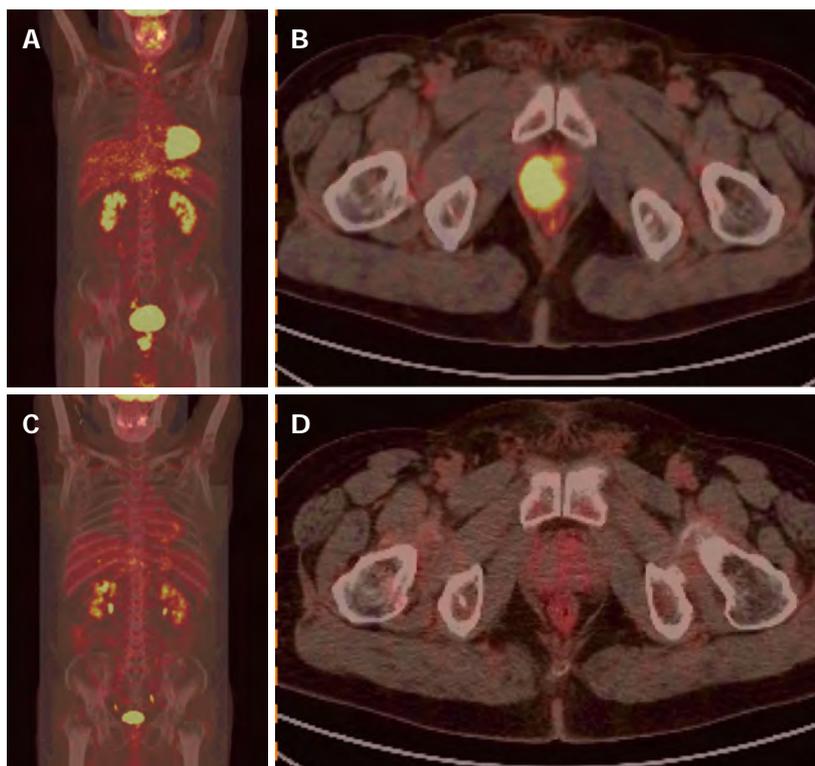
**Key words:** Fluoro-Deoxy-Glucose; Positron emission tomography/computerized tomography; Non-Hodgkin's lymphoma; Prostatic lymphoma; Evaluation

**Core tip:** Primary prostatic lymphoma is extremely rare. There is no consensus on the primary prostatic lymphoma management. We report a case of primary diffuse large B cell lymphoma of the prostate incidentally found by  $^{18}\text{F}$ -fluoro-deoxy glucose (FDG) positron emission tomography/computerized tomography (PET/CT), and finally diagnosed by histopathological examination.  $^{18}\text{F}$ -FDG PET/CT is not only a complement to the conventional imaging studies, but also plays a significant role in the diagnosis and treatment of prostatic lymphoma.

Pan B, Han JK, Wang SC, Xu A. Positron emission tomography/computerized tomography in the evaluation of primary non-Hodgkin's lymphoma of prostate. *World J Gastroenterol* 2013; 19(39): 6699-6702 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i39/6699.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i39.6699>

### INTRODUCTION

Primary prostatic lymphoma is extremely rare, to the best of our knowledge, less than 200 cases have been described in the world literature so far<sup>[1]</sup>, mostly as single case reports, and a few as large-series studies. It constitutes 0.09% of prostate neoplasms and 0.1% of all non-Hodgkin lymphomas<sup>[2]</sup>. The most common pathological



**Figure 1** Positron emission tomography/computerized tomography image. A: Whole body positron emission tomography (PET) image (scan) shows nodal hypermetabolic foci in the prostate, and no abnormally high uptake foci in the other sites of the body; B: The PET/computerized tomography (CT) fusion image displays a nodal hypermetabolic lesion located in the right lobe of the prostate, with maximal standardized uptake value (SUV) being 12.7; C: There were no hypermetabolic foci on the whole body PET imaging following six courses of chemotherapy; D: There was complete remission of the prostatic lesion after six cycles of chemotherapy, and the maximal SUV was 2.0.

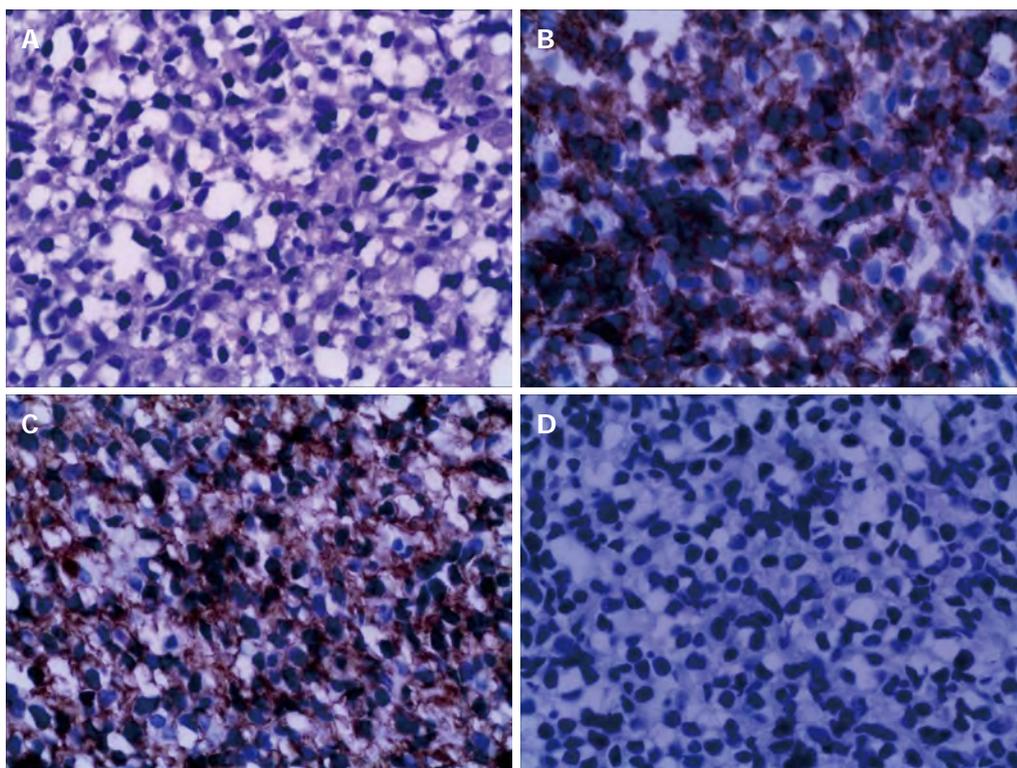
type is diffuse large B cell lymphoma (DLBCL). Because of unspecific clinical symptoms including increased urinary frequency, urinary urgency, occasional hematuria and acute urinary retention, primary prostatic lymphoma is easily misdiagnosed as benign prostatic hyperplasia or prostatitis, especially in elderly men, and it has a poor prognosis<sup>[3,4]</sup>. Serum prostate-specific antigen (PSA) level is usually within normal limits in most patients. Due to the small number of patients reported, there is no consensus on the primary prostatic lymphoma management, including radiotherapy, chemotherapy and radical prostatectomy or combined strategies.

Here we report a case of primary DLBCL of the prostate incidentally found by <sup>18</sup>F-fluoro-deoxy glucose (FDG) positron emission tomography/computerized tomography (PET/CT), and finally diagnosed by histopathological examination.

## CASE REPORT

A 65-year-old man suffered from increased urinary frequency, urinary urgency, urinary endlessness and increased frequency of nocturia for two years. Because of his age and symptoms presenting as benign prostate hyperplasia, he did not take it seriously until the symptoms were aggravating. On examination, the prostate was palpable with degree II of hypertrophy, the median sulcus was shallow, and there was no hepatosplenomegaly. Laboratory findings including blood count, liver and renal function were all normal. Chest X-ray revealed no abnormalities. Serology for human immunodeficiency virus, hepatitis B, and hepatitis C was negative. Tumor markers like alpha-fetoprotein, carcino-embryonic antigen and

total PSA were within normal limits. Ultrasound revealed prostatic hyperplasia and a hypoechoic lesion measuring 3.1 cm × 3.5 cm in size in the right lobe, and the nature of hypoechoic lesion was unidentified. Ultrasound-guided puncture was suggested. The patient was referred for a whole body PET/CT study to identify whether other sites were involved. He was injected with 9.8 millicuries of <sup>18</sup>F-FDG and underwent a whole body scan with a dedicated PET/CT scanner. Except a nodal FDG uptake in the prostate gland with the maximum standardized uptake value (SUV<sub>max</sub>) of 12.7 (Figure 1A and B), all appeared unremarkable. In order to establish the diagnosis, ultrasound-guided transrectal puncture was performed. Biopsy specimens were composed of colorectal mucosal epithelium and aggregated lymphocytes. Immunohistochemical examination demonstrated that CD20 (+), CD79a (+), Pax5 (+), BCL-6 (+), mum-1 (+), and Ki-67 (+, 60%) were positive and CD10 (-), vim (+/-), CD3 (-), CD45RO (-), CD30 (-), CX (-), PSA (-), syn (-), and CGA (-) were negative. Combined morphological and immunophenotyping examinations confirmed a DLBCL of non-germinal center B-cell origin (Figure 2). Myeloproliferation was significant with nuclear shifted to the right, and red-giant cells hyperplasia was also active, and platelets were aggregately distributed, suggesting no evidence of bone marrow involvement. Consequently, Phase I and International Prognostic Index score 1 was considered. He experienced no taboos in chemotherapy, and completed six cycles of mabthera, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) chemotherapy. Three weeks after the end of chemotherapy, the patient underwent PET/CT scan again, which showed complete remission of the prostatic lesion on CT, with



**Figure 2** Combined morphological and immunophenotyping examinations confirmed a diffuse large B cell lymphoma of non-germinal center B-cell origin. A: Intensive proliferation of lymphoid cells (Hematoxylin and eosin,  $\times 400$ ); B and C: Diffuse and consistent expression of CD79a, CD20 (immunohistochemistry,  $\times 400$ ); D: The expression of serum prostate-specific antigen is negative (immunohistochemistry,  $\times 400$ ).

no active foci in the prostatic lesion on PET (Figure 1C, D), and the SUVmax was 2.0.

## DISCUSSION

Primary malignant lymphoma arising from the prostate is notably rare. We use PubMed search method, with key words of primary prostatic lymphoma, 87 Articles were retrieved by the end of Jan 2013, approximately 200 cases reported in the world literature<sup>[1]</sup>. Non-Hodgkin's lymphoma is the major histological subtype of primary Hodgkin's lymphoma (PHL) and DLBCL is the most common. Morphology of lymphoma in this case is typical, diffuse infiltrate lymphoid cells in prostate tissue. Immunohistochemical examination showed diffuse expression of CD79a and CD20 in tumor cell, which are the typical characteristics of diffuse large B cell lymphoma. CD3 being negative and diffuse expression of Mum-1 suggested that tumor cell originated from B cells. Moreover, CD30 being negative also tips for non-Hodgkin lymphoma. Negative CK suggesting non epithelial malignancies, combined with negative PSA, exclude the possibility of prostate cancer. Positive bcl-6 indicates the biological activity of lymphoma, the proliferation fraction was 60% with Ki67 antibody. Combining these conditions together, a diagnosis of non germinal center origin of diffuse large B cell lymphoma was made. The three criteria for the diagnosis of primary prostatic lymphoma were as follows: (1) tumor limited to the prostate only;

(2) the absence of any other lymphoid node or tissue involvement including blood vessels; and (3) a lymphoma free at an interval of at least 1 mo after diagnosis<sup>[5,6]</sup>. Since the clinical symptoms of primary prostatic lymphoma are atypical, including lower urinary tract symptoms, acute urinary retention or hematuria, it is difficult to establish a diagnosis merely based on clinical grounds, which may easily lead to a misdiagnosis as benign prostate hyperplasia or chronic prostatitis. Computerized tomography (CT) and Trans-Rectal UltraSound (TRUS) prostate plays an important role in the location and staging of the primary prostatic lymphoma, it usually manifests as the enlargement of the prostate with low density or hypoechoic lesions with or without abdominal and pelvic enlargement of lymph nodes on CT or US, in addition, CT and TRUS are also of great significance to treatment response to PPL<sup>[7,8]</sup>, Gallium scan is optional when distant lymph node involvement was suspected<sup>[9]</sup>. There were no specific markers for Lymphoma at present, Prostatic Acid Phosphatase May be a sensitive tumor marker protein indicating development and progression of intravascular LBCL, which was a Subtype of diffuse LBCL<sup>[10]</sup>. Tumor marker like PSA usually is within normal range in most patients. The final diagnosis can be established by surgery or transrectal prostate biopsy.

Because of its rarity, there was no consensus on the optimal treatment of prostatic lymphoma at present. A recent study showed that R-CHOP chemotherapy is superior to CHOP and is the first choice for curative intent,

this combination should become the standard for treating DLBCL<sup>[11-13]</sup>. Surgery or radiation is used only for palliative treatment of local symptoms and when the disease is confined to the prostate<sup>[14]</sup>. The prognosis of either primary or secondary prostatic lymphoma is poor and the median survival is around 23 mo after diagnosis<sup>[6]</sup>. If the disease is confined to the prostate, it could be cured with radiotherapy<sup>[3]</sup>.

The use of FDG PET imaging is uncommon for prostatic lymphoma, and only four cases have been reported in the literature: two cases of mucosa-associated lymphoid tissue lymphoma, revealing mild FDG uptake<sup>[15]</sup>, and two cases of DLBCL, which showed intense hypermetabolism<sup>[16,17]</sup>. The extent of FDG uptake may be associated with histological types. In our case, the foci of the prostate demonstrated intense FDG uptake, which was similar to the cases reported.

<sup>18</sup>F-FDG PET/CT helped diagnose the possibility of PPL in this case, and demonstrated the absence of abnormal hypermetabolic foci in any other nodes or organs. <sup>18</sup>F-FDG PET/CT identified significant treatment response after six cycles of chemotherapy.

In conclusion, <sup>18</sup>F-FDG PET/CT is not only a complement to the conventional imaging studies, but also plays a significant role in the diagnosis and treatment of prostatic lymphoma. In short, when a nodal or diffuse uptake is observed in the prostate on PET/CT and PSA is within normal limits, primary prostate lymphoma should be taken into consideration except for prostatic cancer.

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# World Journal of *Gastroenterology*

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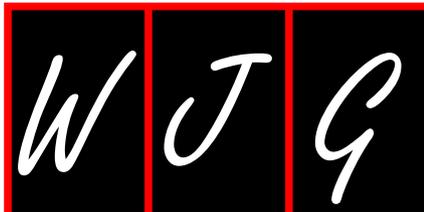
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## Hepatitis C, stigma and cure

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### Abstract

The infection with hepatitis C virus (HCV) is one of the most important global chronic viral infections worldwide. It is estimated to affect around 3% of the world population, about 170-200 million people. Great part of the infections are asymptomatic, the patient can be a chronic carrier for decades without knowing it. The most severe consequences of the chronic infection are liver cirrhosis and hepatocellular carcinoma, which appears in 20%-40% of the patients, leading to hepatic failure and death. The HCV was discovered 25 years ago in 1989, is a RNA virus and classified by the World Health Organization as an oncogenic one. Hepatocellular carcinoma is one of the most important cancers, the fifth worldwide in terms of mortality. It has been increasing in the Occidental world, mainly due to chronic hepatitis C. Hepatitis C is not only a liver disease and a cause of cirrhosis, but also a mental, psychological, familiar, and social disease. The stigma that the infected person sometimes carries is tremendous having multiple consequences. The main cause is lack of adequate information, even in the health professionals setting. But, besides the "drama" of being infected, health professionals, family, society and the infected patients, must be aware of the chance of real cure and

total and definitive elimination of the virus. The treatment for hepatitis C has begun in the last 80's with a percentage of cure of 6%. Step by step the efficacy of the therapy for hepatitis C is rapidly increasing and nowadays with the very new medications, the so called Direct Antiviral Agents-DAA's of new generation, is around 80%-90%.

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**Key words:** Hepatitis C; Chronic; Therapy; Hepatocellular carcinoma; Hepatic cirrhosis; Interferon-alpha; Ribavirin; Social stigma; Depression

**Core tip:** Around 3% of the world population, about 170-200 million people are infected with hepatitis C virus. The chronic consequences of the infection are liver cirrhosis and hepatocellular carcinoma, which appears in 20%-40% of the patients. Hepatitis C is not only a liver disease but also a mental, psychological, familiar, and social disease. The stigma that the infected person sometimes carries is tremendous. But, besides the "drama" of being infected, health professionals, family, society and infected patients, must be aware of the chance of real cure and definitive elimination of the virus. Step by step, the efficacy of the therapy for hepatitis C is rapidly increasing and with the new medications, the Direct Antiviral Agents-DAA's, is around 80%-90%.

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### HEPATITIS C AND CURE

#### Step by step increasing the efficacy

Hepatitis C is one of the most important global chronic infection worldwide: it is estimated to affect 170-200

million people, while chronic hepatitis B and human immunodeficiency virus (HIV) infection affects respectively 350 million and 34 million. There is no vaccine for hepatitis C. Hepatitis B is easily preventable by vaccination. Another characteristic of hepatitis C virus (HCV) infection is the high risk of evolution to chronicity, more than 50% which can be around 80% in same series<sup>[1]</sup>. Another characteristic is the absence of symptoms for decades before the phase of decompensation of liver cirrhosis and the appearance of hepatocellular carcinoma.

The most severe chronic consequences of the infection are liver cirrhosis and hepatocellular carcinoma, leading to hepatic failure and death, which can appear in 20%-40% of the patients<sup>[2]</sup>.

In effect, cirrhosis is the end-stage of every chronic liver disease. Its natural history is characterized by an asymptomatic phase, called “compensated” followed after several years or decades by a “decompensated” phase. The patient, in the decompensated phase has a median of survival of 2 years<sup>[3]</sup>.

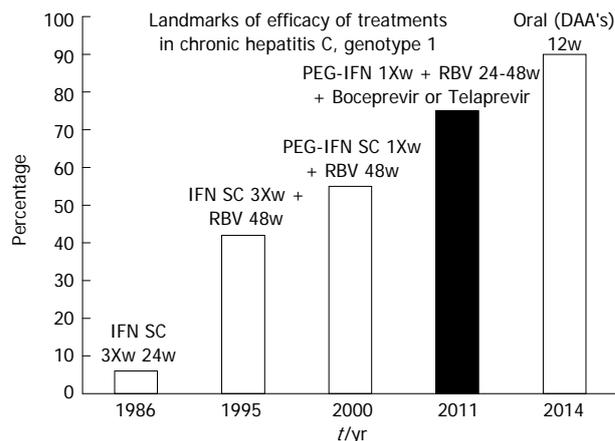
This phase can be characterized by a rapid clinical evolution with all the complications of a cirrhotic liver with portal hypertension: ascites, sepsis (the majority from spontaneous bacterial peritonitis), varices bleeding, jaundice, mental alterations (encephalopathy), renal failure (hepatorenal syndrome), caquexia<sup>[4,5]</sup>.

Liver cirrhosis is one of the most oncogenic situations in medical terms. The development of hepatocellular carcinoma (HCC) is a real fact, occurring in 1%-4% each year and is becoming in some centers the most frequent complication of HCV cirrhosis<sup>[6]</sup>.

The quality of life in the decompensated phase can be very poor with frequent hospitalizations and readmissions<sup>[7]</sup>. At this stage only liver transplantation is really effective for median or long term survival. But if the virus is still active, the reinfection is almost universal<sup>[8]</sup>. But, besides the “drama” of being infected, health professionals, family, society and infected patients, must be aware of the chance of real cure and total and definitive elimination of the virus<sup>[9]</sup>.

The HCV was discovered 25 years ago in 1989, is a RNA virus and classified by the World Health Organization as an oncogenic one<sup>[10]</sup>. The discover of the virus has open the way to a diagnosis test (anti-HCV) and several studies of molecular biology, virology and pharmacology<sup>[11]</sup>. The treatments for hepatitis C has begun in the last 80's with a percentage of cure of 6%<sup>[12]</sup>. Step by step the efficacy of the therapy for hepatitis C is rapidly increasing and nowadays with the very new medications, the oral Direct Antiviral Agents-DAA's, is around 80%-90%<sup>[13]</sup>.

The therapy of hepatitis C is an example of the capacity of modern medicine to translate the basic research to the clinical setting (Figure 1). Several types of medications have been used in to treat hepatitis C throughout these 25 years of success: first, human interferon (INF, three times weekly, 6% of efficacy), in 1995 Ribavirin has appeared to be used in combination with INF (34%-42% of efficacy), in 2001 Pegylated INF once weekly with



**Figure 1** Percentage of cure of hepatitis C genotype 1. IFN: Interferon; RBV: Ribavirine; PEG: Peginterferon; DAA: Direct antiviral agents.

ribavirin (45% of cure for genotype 1 and 70%-80% for genotype 3)<sup>[14]</sup>. In 2011 another step with the combination of Peginterferon and Ribavirin with two Protease inhibitors, region 3/4 (Boceprevir<sup>[15]</sup> and Telaprevir<sup>[16]</sup>), the triple therapy, leading to cure in 70%-80% of cases.

Several clinical trials are in rapid development worldwide with the new DAA's (Direct Acting Antiviral Agents) interacting with several of vital components of the virus (NS 3/4, NS5A, NS5B Polymerase, *etc.*). In effect a new generation medications is rapidly approaching, like Sofosbuvir<sup>[17]</sup>, Daclastavir, Asunaprevir<sup>[17]</sup>, ABT-450<sup>[18]</sup>, Faldaprevir, Simeprevir, Deleobuvir, some of them only using oral agents, for a period of 12 wk, with a few negligible side effects, having a chance of viral eradication of 80%-90%.

In fact, in a quarter of a century, the percentage of cure has increased from 6% to 90% of cure. From three injections a week during 48 wk to some pills a day during 12 wk! Another important development that has positively affected the quality of life of patients, allowing access to treatment and possible cure is the Transitory Elastography (Fibroscan<sup>®</sup>). Is an ultrasonographic device with very good acuity in the diagnosis of liver fibrosis, mainly when there is liver cirrhosis. The number of liver biopsies has decreased<sup>[19]</sup> in some centers around 90%.

The efficacy of the therapy is assessed by an important finding, *i.e.*, the viral load: RNA HCV (by a sensitive test) must be negative 24 wk after stopping therapy. If this happens, more than 99% of patients will never be positive again.

### Cure of hepatitis C

It is the only global chronic viral infection that is possible to cure. The other ones are hepatitis B and HIV infecting respectively 350 and 34 million people worldwide but with no chance of cure in chronic cases.

In the beginning of this story of success, the medical community was afraid of the word cure. But now it is well known this word can be used with property but with some restrictions. In effect is a virological cure for

**Table 1 Benefits of cure of hepatitis C**

Benefits of cure of hepatitis
1 Negative HCV RNA (viral load) for life, in more than 99% of cases
2 Negative HCV RNA in the liver
3 HCV RNA negativation in PBMC
4 No detection of the genotype
5 Sometimes, a few year later, the anti-HCV test can became negative, the so called "seroreversion"
6 Normalization of aminotransferases (AST, ALT) and GGT
7 Changing of ultrasound findings (contours can became regular, reduce of diameter of portal vein in case of portal hypertension)
8 Disappearance of the lymphnodes near the liver (helium)
9 Decrease of the values for Elastography (Fibroscan®)
10 Reducing the risk of progression to cirrhosis
11 Reversion of cirrhosis in some cases
12 Disappearance of oesophageal varices
13 Reducing the risk of progression to liver cancer
14 Reduced risk of decompensated liver disease (ascites, jaundice, rupture of oesophageal varices, encephalopathy)
15 Reducing to zero the risk of recurrence after liver transplantation (if necessary)
16 Improved quality of life (asthenia, fatigue, general well-being)
17 Reducing of the psychological impact (anxiety/depression)
18 Disappearance of the risk of sexual transmission
19 Disappearance of the risk of perinatal transmission
20 Decrease in the insurance premium
21 Cure of associated conditions (porphyria cutanea tarda, polyneuropathy, urticaria, cryoglobulinemia, splenic lymphoma)
22 Reducing personal, family and social stigma
23 The treatment is proved cost-effective
24 Benefit to public health
25 Reduced risk of death from liver disease
26 Neurocognitive improvement
27 Cure of hepatitis C

Benefits of virological response (HCV RNA negative 24 wk after finishing therapy). HCV: Hepatitis C virus; PBMC: Peripheral Blood Mononuclear Cells; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase.

life. It is proved that virus is not detected on liver cells or mononuclear blood cells. Nor there is an occult disease as is the case for chronic hepatitis B (HBV DNA negative or with low levels, having HBsAg negative and a risk of relapse in case of immunosuppression).

Albeit is a definitive one, we must be cautious in patients having liver cirrhosis, because the chance of development of hepatocellular carcinoma is strongly reduced, but still remains. It is one of the reasons to treat patients with mild or moderate fibrosis, in order to reduce the chance, while in a stage of a less severe disease, of evolution to cirrhosis and hepatocellular carcinoma. Liver cirrhosis, *per se*, is a disease having a risk of 1%-4% per year of evolution to hepatocellular carcinoma.

The benefits of cure are tremendous. Hepatitis C should be considered a global disease. As for the definition of Health of World Health Organization, ("Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity") chronic hepatitis C is a physical, mental and social disease, affecting globally the individual, the couple, the family, and the society as a whole.

There are some myths about hepatitis C: "almost always lethal, more severe than acquired immunodeficiency

syndrome (AIDS), very contagious disease, not curable, the adverse events of therapy are huge and very severe, *etc.*" But the benefits of cure, in global terms considering the physical, mental and social aspects are several (Table 1).

### **Mental health and quality of life in hepatitis C**

Besides the natural history of this disease, the personal impacts of a diagnosis of hepatitis C infection and its treatment strongly affect the patients' quality of life<sup>[20-22]</sup>. Mental health problems frequently occur in chronic infection with HCV and during the antiviral treatment. These individuals frequently present neuropsychiatric symptoms like fatigue, anxiety, depression and cognitive disorders<sup>[23,24]</sup>.

Regarding neuropsychiatric symptoms, one can identify two distinctive patterns in its relation with HCV infection. On one hand, individuals with chronic hepatitis C have higher prevalence of psychiatric disorders, including depression<sup>[25]</sup>. On the other hand, individuals with psychiatric records present higher HCV infection rates than the average population<sup>[26]</sup>.

A combined therapeutics using Pegylated IFN is commonly used in these patients and proves to be a fundamental and consensual intervention for a favourable change in the natural history of the disease<sup>[27,28]</sup>.

However, this treatment is associated with a high number of adverse reactions like: irritability, insomnia, fatigue and loss of appetite. Apart from these, neuropsychiatric symptoms (especially depression, and sometimes with suicidal ideation) are among the most common secondary effects in therapeutics with IFN, being one of the main causes why patients interrupt their treatment<sup>[24,29,30]</sup>. It is noteworthy that, up to a certain extent, psychopathologic symptoms (depression, cognitive disorders) may be associated to HCV infection, even without an INF treatment, and may be related to direct HCV neurotoxicity<sup>[29-32]</sup>.

A large number of patients undergoing treatment for VHC infection should be referred for psychiatric evaluation and, if necessary, should received treatment for depression and other neuropsychiatric symptoms.

Recognition of depression and other neuropsychiatric symptoms is important, and could improve adherence to VHC treatment. This symptomatology negatively affects the individual's perceived quality of life, its general functioning, work capacities, less participation in life and medical care, increase mobility and mortality, decrease overall well-being and quality of life<sup>[27,33]</sup>. Furthermore, therapeutic used in HCV treatment is associated to impairment in all of these dimensions<sup>[34]</sup>.

Thus, considering the impact in patients' mental health, before and during the treatment, an interdisciplinary approach should be followed and encouraged when dealing with HCV infected patients<sup>[24]</sup>.

### **Stigma and hepatitis C**

Diagnosis with hepatitis C was reported to have pro-

found impacts on social functioning. Perceived stigma associated with HCV infection leads to high levels of anxiety and exaggerated fear of transmission, and it can be a major cause of social isolation and reduced intimacy in relationships<sup>[35]</sup>.

Epidemiological studies suggest that more than 90% of transmission in developed countries takes place through the sharing of non-sterilized needles and syringes in the intravenous drug-using population<sup>[36]</sup>.

Because the vast majority of people with hepatitis C have a history of intravenous drug use, they are frequently blamed for acquiring the disease, and viewed as irresponsible, accountable and “unworthy”<sup>[37]</sup>. Furthermore, as a blood-borne disease, hepatitis C is strongly associated with HIV. This association exists due to the fact that intravenous drug abuse is a significant risk factor for contracting both diseases and this can be a stigmatizing factor for this patients<sup>[38]</sup>.

Stigma can be defined as attitudes expressed by a dominant group, which views a collection of others as socially unacceptable. The notion of stigma denoting shameful relations and deviations from what is considered “normal” has a long history within infectious disease, in particular HIV<sup>[39]</sup>, and more recently in hepatitis C infection.

These norms, behaviours and beliefs surrounding hepatitis C infection can lead to alienation from family and friends, as well as to discrimination (perceived or real) in health services and workplaces<sup>[40]</sup>.

Stigma can affect self-esteem and quality of life. It can also impede the success of diagnosis and treatment, leading to continuing risk of disease transmission. It is a social phenomenon that influences the course of illness and marginalizes patients<sup>[41]</sup>.

Since stigmatization affects not only the individual but also the whole course of the disease, health care workers are not immune to stereotypes and judgements that might influence the course of the treatment of HCV patients. Changing this behaviour will help prevent patients’ isolation, withdrawal of treatment and it will increase the search for medical help<sup>[42]</sup>.

Hepatitis C should have a global approach in its treatment. It requires broad-based educational efforts in order to increase the understanding of this disease, still connected to several pejorative stereotypes<sup>[42]</sup>. These efforts should include patients and their family, health care providers and the society as a whole. Further knowledge of hepatitis C stigma is central to assisting patients in self-managing their illness, and it is important to reduce the disease burden.

### Several benefits of cure hepatitis C

The goal of therapy is to eradicate HCV infection. The endpoint of therapy is sustained virological response (SVR). Once obtained, SVR usually equates to cure of infection in more than 99% of patients<sup>[43]</sup>.

If the test for assessing viral load is negative 24 wk after finishing therapy, we can talk of “cure”. With the new

treatments, the oral DAAs, the assessment of sustained viral response can be shortened to 12 wk after ending the treatment. HCV RNA detection and quantification should be made by a sensitive assay (lower limit of detection of 50 IU/mL or less), ideally a real-time PCR assay. If the patient has already cirrhosis, the risk of develop hepatocellular carcinoma still persist for some years and deserves an abdominal ultrasound every six months.

When the SVR is obtained the global benefit is as it follows: if there is no another reason for AST, ALT or GGT elevation, namely alcohol consumption or obesity, they became persistently normal.

At virological level, the HCV RNA is no longer detected in the liver<sup>[44]</sup> or even in the Peripheral Blood Mononuclear Cells (PBMC) by sensitive tests<sup>[45]</sup>. The genotype becomes and remains negative, because there is no virus for detection.

One of the things that can cause some confusion is the fact that the anti-HCV (a marker of past infection) generally remains positive. The index can decrease slowly and the anti-HCV can become negative, several years after, as it happens in the context of acute hepatitis C<sup>[46]</sup>.

On the Hepatic Elastography (Fibroscan<sup>®</sup>) the values generally decrease along some months<sup>[47]</sup>. At the abdominal ultrasound the findings can change: the liver contours can become regular, the diameter of portal vein reduces in case of presence of portal hypertension. The disappearance of the lymph nodes in the hepatic hilum can be a finding<sup>[48]</sup>. There are some studies showing that dimensions of these lymph nodes are related with the levels of viral replication<sup>[49]</sup>.

Regarding liver disease the risk of progression to cirrhosis decreases. In some cases it occurs a reversion of cirrhosis<sup>[50]</sup>. In fact this is no longer an irreversible situation as was thought some years ago; the disappearance of oesophageal varices is also fact<sup>[51]</sup>. On the other side, there is a decrease of risk of evolution for more severe stages of liver disease like the progression hepatocellular carcinoma<sup>[52]</sup> and risk of the decompensated liver disease (ascites, jaundice, rupture of oesophageal varices, encephalopathy, jaundice, *etc.*)<sup>[53]</sup>.

In the case that a liver transplantation would be necessary the risk of reactivation is no longer present. More than 95% of cases of patients transplanted for cirrhosis associated with HCV will have again HCV RNA positive and 50% will develop severe forms of liver disease a few years after transplant<sup>[54]</sup>. Because of that, to treat patients with cirrhosis or intense fibrosis must be done as soon as possible.

There is an improvement of quality of life<sup>[55]</sup> (asthenia, fatigue, general well-being, *etc.*) assessed by adequate tests and more important on the mental level there is a reduction of the psychological impact (anxiety and/or depression).

In strong relation to cure, the risk of sexual<sup>[56]</sup> and perinatal transmission<sup>[57]</sup> disappear. These are very important advantages of SVR in the treatment of hepatitis C. We must not forget, that hepatitis C, besides a liver

disease is also an infectious and transmissible disease. We can consider the cure as “belonging” to the patient himself but also to his family, his couple, *etc.* Is also a familiar disease. There are some patients who don't tell the family or to the couple afraid of the consequences of the bad news. If patients insist with Insurance Companies they must decrease the insurance premium because there is less risk of clinical evolution.

There are reports of the control and even disappearance of some associated conditions like porphyria cutanea tarda<sup>[58]</sup>, polyneuropathy<sup>[59]</sup>, urticaria<sup>[60]</sup>, cryoglobulinemia<sup>[61]</sup>, splenic lymphoma<sup>[62,63]</sup>.

Depending on the countries and the burden of the infection, the treatment was proved to be cost-effective<sup>[64]</sup>. Reducing of personal, psychological, family and social stigma is a huge benefit for all. Stigma is a fact that must be considered in the setting of HCV therapy and also when considering the real burden of the disease.

## CONCLUSION

Considering the global approach we can consider that to cure HCV chronic infection is a real benefit to public health mainly by reducing the risk of complications and dying because of liver disease. Having access to the most modern therapies, the disease is almost a curable disease and the efficacy of treatment markedly increases the survival of patients infected. Chronic hepatitis C is a silent epidemic, a global disease with a strong stigma, but with high chance of definitive cure<sup>[65]</sup>.

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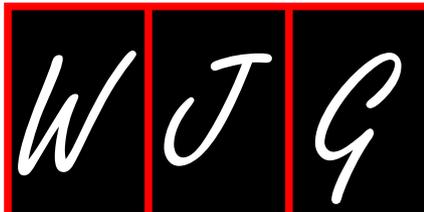
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## ***Clostridium difficile* infection in the community: Are proton pump inhibitors to blame?**

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**Core tip:** Population-based studies demonstrate that non-antibiotic associated, community-acquired *Clostridium difficile* infection (CDI) is increasingly common. Patients with community-acquired CDI are younger and have fewer comorbidities compared to patients with hospital-associated CDI. Proton pump inhibitors may be a risk factor for non-antibiotic associated, community-acquired CDI.

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### Abstract

Once a nosocomial disease, *Clostridium difficile* infection (CDI) now appears frequently in the community in the absence of exposure to antibiotics. Prior studies have shown that patients with community-acquired CDI are younger, more likely to be female, and have fewer comorbidities compared to patients with hospital-associated CDI. Because most studies of CDI are hospital-based, comparatively little is known about community-acquired CDI. The recent study by Chitnis has received widespread attention because it used active surveillance to capture all cases of community-acquired CDI within a large population and assessed key risk factors. The authors found that low-level healthcare exposure and proton pump inhibitor use were common among those with non-antibiotics associated, community-acquired CDI. In this commentary, we discuss the changing epidemiology of community-acquired CDI and the evidence basis for the controversial association between proton pump inhibitors and community-acquired CDI.

### COMMENTARY ON HOT TOPICS

*Clostridium difficile* (*C. difficile*) infection (CDI) is the most feared gastrointestinal epidemic in the developed world with increasing incidence, virulence, and case fatality rates<sup>[1-5]</sup>. Formerly a nosocomial disease, CDI has become common in the community<sup>[6,7]</sup>. Early reports suggested that the risk factors associated with community-acquired CDI differ from the traditional risk factors associated with nosocomial CDI with relatively young and healthy individuals affected<sup>[8]</sup>. Thus we read with great interest the recent article by Chitnis *et al*<sup>[9]</sup> describing a multi-center study of the factors associated with community-acquired CDI.

### COMMUNITY-ACQUIRED *CLOSTRIDIUM DIFFICILE* INFECTION

Pseudomembranous colitis was reported in the 19<sup>th</sup> cen-

tury and has long been understood as an antibiotic-associated phenomenon<sup>[10,11]</sup>. In 1978, *C. difficile* was identified as the causative agent of disease; subsequent reports recognized that pseudomembranous colitis caused by *C. difficile* could occur without antibiotics<sup>[12]</sup>. But it was only beginning in 2005 that it became understood that *C. difficile* infection was frequently occurring in the community<sup>[7]</sup>.

Community-acquired CDI differs from hospital-associated disease, although many uncertainties remain. In the United States and Europe, 15%-44% of CDI occurs in the community without an identifiable antecedent healthcare exposure<sup>[8,13-15]</sup>. Compared to individuals with nosocomial CDI, those with community-acquired CDI are younger, have fewer comorbidities, and are more likely to be female<sup>[7]</sup>. Most surprisingly, patients with community-acquired CDI often do not report exposure to antibiotics<sup>[16]</sup>.

If antibiotics are not essential in community-acquired CDI, what are the crucial risk factors? This question has been difficult to answer, in part because it is challenging to study community-acquired CDI in the United States. Cases of CDI arising in the community rarely require hospital admission. However, many studies of community-acquired disease are hospital-based and thus miss a large proportion of disease that both arises and is treated in the community<sup>[17-21]</sup>. In 2009, to address this problem, the Centers for Disease Control and Prevention (CDC) began a population-based program of active surveillance encompassing 11 million people<sup>[22]</sup>. Working with laboratories within the active surveillance area, all newly positive *C. difficile* stool tests were prospectively identified. Based on interviews with affected individuals, cases were classified as hospital-associated (defined as diarrhea and stool collected > 3 d to < 12 wk from a hospitalization) or community-acquired (all other cases). Community-acquired cases were assessed for risk factors including use of antibiotics or proton pump inhibitors (PPIs) and healthcare exposures within the previous 12 wk (classified as high-level exposure for dialysis or emergency department visits or low-level exposure for visits to a physician's office).

Chitnis *et al*<sup>[9]</sup> report on the first results of this valuable project. The authors identified 984 patients with confirmed community-acquired *C. difficile* infection. Patients were relatively young (median age 51 years old) and predominantly female (67%). Yet morbidity and mortality were surprisingly high. One quarter of patients with community-acquired CDI were hospitalized for treatment and there was a 6% combined rate of death, colectomy, or admission to an intensive care unit. Overall, 41% of patients reported a high-level healthcare exposure, 41% of patients reported a low-level healthcare exposure, and 18% of patients reported no healthcare exposure. Sixty-four percent of patients recalled antibiotic use within the preceding 12 wk. Compared to patients who reported recent antibiotic use, those that did not report antibiotic use were slightly more likely to report PPI use (31% *vs* 26%) but not histamine 2-receptor antagonist use (10%

*vs* 9% respectively). The study has no comparison group so its most important findings are essentially descriptive. Nonetheless, the concerning implication is that non-antibiotic associated CDI is rising. Are PPIs to blame?

## CLOSTRIDIUM DIFFICILE INFECTION AND PPIs

Over thirty observational studies and multiple meta-analyses indicate that PPIs are a risk factor for *C. difficile* infection<sup>[17,18]</sup>. Citing these findings in 2012, the United States Food and Drug Administration issued a warning regarding increased risk of CDI among patients taking long term PPIs<sup>[23]</sup>. Yet many questions remain regarding the relationship between PPIs and *C. difficile*. The data connecting PPIs and CDI is observational. Because patients who are prescribed PPIs differ in many ways from those who are not prescribed PPIs<sup>[24,25]</sup>, it is possible that the observed association between PPIs and CDI is attributable to unmeasured confounding<sup>[26]</sup>. And there is comparatively little data that specifically addresses PPIs in community-acquired CDI.

There are a few reasons to suspect that the relationship between PPIs and CDI might be different among those with community-acquired compared to hospital-associated CDI. First, the highly toxigenic North American pulsed-field 1 (NAP1) strain has been linked to hospital-associated<sup>[3,27]</sup> rather than community-acquired cases; it is possible that the relationship between PPIs and CDI is affected by *Clostridial* strain. Second, a potential mechanism by which PPIs increase risk for CDI may be via alteration of the colonic microbiome<sup>[28-31]</sup>. Thus hospitalized patients, who can have altered microbiomes compared to those in the community<sup>[32]</sup>, may be affected differently by PPIs. Finally, antibiotic exposure, which differs between hospitalized and non-hospitalized patients, may modify the relationship between PPIs and CDI<sup>[33]</sup>.

So what is the evidence that PPIs are a risk factor for CDI in the community? Only a handful of studies include disease that is both acquired and treated in the community. A large, population-based study conducted within a United Kingdom dataset identified over 1000 cases of community-acquired CDI from 1994 to 2004<sup>[34]</sup>. The authors found that only 37% of cases had been prescribed antibiotics within the previous 90 d; compared to matched controls, patients prescribed PPIs within the previous 90 d had a nearly 3-fold increased risk for CDI. A Scottish study conducted among adults  $\geq$  65 years old identified all cases of community-acquired CDI<sup>[35]</sup>. After adjusting for covariables, the authors found that patients prescribed PPIs within the previous 6 mo had a 1.7-fold increased risk for CDI compared to matched controls. Finally, a study using a large United States insurance claims database identified all cases of CDI from 2004 to 2007 in Iowa and South Dakota<sup>[13]</sup>. Seventy-three percent of cases had been prescribed antibiotics within the previous 180 d; patients prescribed PPIs or histamine-2 receptor antagonists within the previous 180 d had a 2.3-fold increased

risk for community-acquired CDI compared to matched controls. These findings imply that the association between PPIs and CDI is at least as strong in community-acquired disease as in its more familiar hospital-associated form.

The study by Chitnis *et al*<sup>[9]</sup> was not designed to directly test the hypothesis that PPIs are associated with CDI in the community. Instead, this study yields valuable lessons regarding the epidemiology and risk factors for community-acquired *C. difficile* infection. Using active surveillance to capture all cases of community-acquired CDI, the authors have shown that non-antibiotic associated, community-acquired CDI is common, and that affected patients frequently have some form of healthcare exposure that falls short of actual hospitalization. Overall, rates of PPI use were extraordinarily high, nearly 30% among patients with community-acquired CDI compared to less than 3% in the general population<sup>[36]</sup>. Future studies should test the hypothesis that PPIs are a risk factor for non-antibiotic associated, community-acquired *C. difficile* infection and assess whether interventions causing decreased PPI use can also decrease rates of CDI. For now, the findings of Chitnis *et al*<sup>[9]</sup> highlight the fact that community-acquired CDI is a very real problem and remind us that PPIs should be prescribed only in situations where they are indicated.

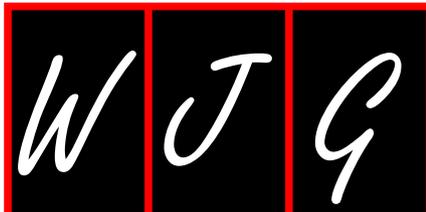
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WJG 20<sup>th</sup> Anniversary Special Issues (2): Hepatitis C virus

## Hepatitis C and pregnancy

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### Abstract

Acute hepatitis C is a rare event in pregnancy. The most common scenario is chronic hepatitis C virus (HCV) infection in pregnancy. During pregnancy in women with chronic HCV infection a significant reduction in mean alanine aminotransferase levels has been reported, with a rebound during the postpartum period. In few cases exacerbation of chronic hepatitis C has been reported in pregnancy. A cofactor that might play a role in the reduction of liver damage is the release of endogenous interferon from the placenta. Observations regarding serum HCV-RNA concentration have been variable. In some women HCV-RNA levels rise toward the end of pregnancy. In general, pregnancy does not have a negative effect on HCV infection. Conversely, chronic hepatitis does not appear to have an adverse effect on the course of pregnancy, or the birth weight of the newborn infant. The role of spontaneous abortion is approximately the same as in the general population. The overall rate of mother-to-child transmission for HCV is 3%-5% if the mother is known to be anti-HCV positive. Co-infection with human immunodeficiency virus (HIV) increases the rate of mother-to-child transmission up to 19.4%. Numerous risk factors for vertical transmission have been studied. In general, high viral load defined as at least  $2.5 \times 10^6$  viral RNA copies/mL, HIV co-infection, and invasive procedures are the most important factors. Both interferon and ribavirin are contraindicated

during pregnancy. Viral clearance prior to pregnancy increases the likelihood that a woman remains non-viremic in pregnancy with a consequent reduced risk of vertical transmission.

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**Key words:** Hepatitis C virus; Pregnancy; Virus transmission; Liver damage; Viral RNA

**Core tip:** In general, pregnancy does not have a negative effect on hepatitis C virus (HCV) infection. Conversely, chronic hepatitis does not appear to have an adverse effect on the course of pregnancy, or the birth weight of the newborn infant. The overall rate of mother-to-child transmission for HCV is 3%-5% if the mother is known to be anti-HCV positive. Co-infection with HIV increases the rate of mother-to-child transmission up to 19.4%.

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### INTRODUCTION

The global prevalence of hepatitis C virus (HCV) infection is 2%-3%, with 130-170 million HCV-positive people - most of them chronically infected<sup>[1]</sup>. The epidemiology of HCV is varies among countries and the reported prevalence of HCV in pregnant women has not been extensively studied, due to the lack of preventative screening of infection and the lack of preventative measures of mother-to-child transmission. The pathogenesis of HCV infection during pregnancy remains poorly understood. During pregnancy, the maternal immune system must at the same time develop tolerance to paternal alloantigens to prevent maternal immune aggression against the fetus

**Table 1** Prevalence of serum anti-hepatitis C virus and hepatitis C virus RNA in in studies examining  $\geq 3000$  pregnant mothers

Ref.	No. screened	Anti-HCV <sup>+</sup>	HCV RNA <sup>+</sup>
Hillemanns <i>et al</i> <sup>[3]</sup>	3712	0.94%	57%
Conte <i>et al</i> <sup>[4]</sup>	15250	2.40%	72%
Ward <i>et al</i> <sup>[5]</sup>	4729	0.80%	72%
Baldo <i>et al</i> <sup>[6]</sup>	4008	1.70%	42.50%
Goldberg <i>et al</i> <sup>[7]</sup>	3548	0.60%	NA
Kumar <i>et al</i> <sup>[8]</sup>	8130	1.03%	NA
Sami <i>et al</i> <sup>[9]</sup>	5902	1.80%	NA
Ohto <i>et al</i> <sup>[10]</sup>	22664	0.50%	54%
Seisededos <i>et al</i> <sup>[11]</sup>	474539	0.15%	NA
Ugbebor <i>et al</i> <sup>[12]</sup>	5760	3.60%	NA
Pinto <i>et al</i> <sup>[13]</sup>	115386	0.10%	NA
Urbanus <i>et al</i> <sup>[14]</sup>	4563	0.30%	NA
Mean		1.16%	59.75%

HCV: Hepatitis C virus; NA: Not available.

and maintain active immunity against HCV to protect both mother and fetus from infection<sup>[2]</sup>. Moreover, it is important to define the effect of pregnancy on HCV and vice versa. Finally, understanding the transmission of HCV to infants and the risk factors correlated with transmission will provide information for counseling and management of HCV infection during puerperium.

### EPIDEMIOLOGY OF HCV IN PREGNANCY

The current prevalence of HCV in pregnant mothers is difficult to be estimated, because of the lack of selective screening in a large proportion of this population. The prevalence of serum HCV antibody in cohorts of  $\geq 3000$  pregnant mothers ranges between 0.1% and 3.6% with a mean of 1.16%, and the prevalence of HCV RNA ranges between 42.5% and 72%<sup>[3-14]</sup> (Table 1). It is particularly interesting that in Egyptian rural villages the prevalence of anti-HCV reaches a mean of 15.5%, with a 30% rate in women aged  $> 35$  years<sup>[15]</sup>. The epidemiological risk factors explaining this dramatic high prevalence include: previous delivery attended by a traditional birth assistant; circumcision by a traditional birth assistant or a “healthy barber”; and injection therapy for schistosomiasis.

In all series the main route of acquiring HCV in pregnant mothers is intravenous drug use, whereas a previous blood transfusion is a risk factor in up to 11% of cases<sup>[4,6,16-22]</sup> (Table 2).

It should be noted that a large study in Japan has demonstrated a significant decline in HCV prevalence in pregnant women<sup>[10]</sup>. In a cohort studied between May 1990 and November 2004, a total of 22664 consecutive serum samples were screened for anti-HCV. Among women known to be transfused, rates fell from 14.8% to 3.1% with the implementation of HCV screening. In non-transfused women rates fell from 1.8% to 0.3%. This reduction has been mainly explained by the hygienic improvements including needles for medical injections and single-use acupuncture needles. The prevalence of HCV infection has been found to be lower in recent years com-

**Table 2** Proportion of intravenous drug use or blood transfusion risk factors in pregnant hepatitis C virus-positive mothers

Ref.	No. screened	IVDU	BT
Paccagnini <i>et al</i> <sup>[16]</sup>	70	79.00%	9.00%
Zanetti <i>et al</i> <sup>[17]</sup>	94	17.00%	13.00%
Granovski <i>et al</i> <sup>[18]</sup>	147	79.00%	NA
Hillemanns <i>et al</i> <sup>[19]</sup>	35	20.00%	11.00%
Resti <i>et al</i> <sup>[20]</sup>	403	25.00%	4.50%
Gervais <i>et al</i> <sup>[21]</sup>	26	35.00%	19.00%
Conte <i>et al</i> <sup>[4]</sup>	370	32.00%	18.00%
Baldo <i>et al</i> <sup>[6]</sup>	40	45.00%	2.50%
Mast <i>et al</i> <sup>[22]</sup>	242	52.30%	19.80%
Mean		42.60%	12.10%

IVDU: Intravenous drug use; BT: Blood transfusion.

pared to the 1990s and 2000s, even in Upper Egypt<sup>[23]</sup>. The lower prevalence rates may be due to the amelioration of standard care and to the hepatitis B virus (HBV) vaccination of all newborns, together with the mandatory testing of blood donors and blood products and careful preoperative measures.

### ACUTE HEPATITIS C

Acute hepatitis C during pregnancy has been rarely reported in pregnancy, and limited to high-risk groups, such as intravenous drug users, whereas the risk in the general population is negligible. Flichman *et al*<sup>[24]</sup> described a case in a 16-year-old pregnant woman who developed icteric acute hepatitis. She was a chronic HIV carrier and was diagnosed with HCV superinfection. HCV infection interfered with HIV replication, because the HIV levels were undetectable during the course of acute HCV infection. Another case of acute hepatitis C was described in a 26-year-old woman at week 16 of pregnancy, who was treated with a short course of interferon<sup>[25]</sup>. Although the therapy was discontinued due to adverse effects, a complete biochemical and virological response was obtained. Premature labor occurred and healthy twin infants were born transvaginally; they did not acquire HCV infection. A case with acute hepatitis C and premature delivery, but no vertical transmission has also been reported<sup>[26]</sup>. Finally, acute hepatitis C was reported in a 29-year-old Japanese woman who developed fatigue, jaundice and ascites after a needle-stick injury<sup>[27]</sup>. When these symptoms were presented, the patient became pregnant by artificial insemination. She was treated with interferon- $\beta$  following eradication of HCV infection without severe side effects. In general, however, it should be said that acute hepatitis C is a rare event in pregnancy and that there is also a publication bias, due to reporting only severe cases<sup>[28]</sup>.

From the epidemiological point of view, however, acute hepatitis C is not a relevant problem in pregnancy<sup>[29,30]</sup>.

The differential diagnosis of acute hepatitis C requires us to rule out hepatitis caused by other hepatotropic viruses (hepatitis A, B, D and E) and liver disorders unique to pregnancy. Hepatitis A is transmitted by the oral-fecal

route. The whole Mediterranean area is endemic for this infection. Acute hepatitis A virus infection in pregnancy has been described only in anecdotal cases, and virtually in 100% of cases it has a favorable outcome<sup>[31]</sup>. To date, acute episodes of HBV infection in Italy have been rare, because HBV vaccination in newborns and adolescents has been compulsory since 1991. However, acute infections in pregnancy can occur in immigrants from developing countries or in high-risk groups, such as intravenous drug users. The clinical course of acute hepatitis B in pregnancy is benign, and the risk of fulminant cases is similar in pregnant and non-pregnant women. Acute infection with hepatitis D virus (HDV) does not represent a relevant risk for death in pregnancy. However, HDV epidemiology has dramatically changed over the past 20 years. Only sporadic cases are recorded in the Mediterranean countries, whereas, it is still a problem in some South American areas (especially in the Amazon Basin) and India<sup>[32]</sup>. Hepatitis E virus (HEV) is endemic in many tropical and subtropical countries, including Central Asia and India<sup>[33]</sup>. Sporadic cases in immigrants from these countries might be seen in developed countries. Generally, symptoms of acute illness including jaundice develop in 80% of cases. The reported risk for fulminant hepatic failure in pregnancy was up to 20% in previous studies<sup>[34]</sup>. Even in mothers who recover from acute hepatitis, an increased frequency of abortion and fetal complications have been reported. Moreover, the severity of acute hepatitis E in pregnant women seems directly correlated with viral load<sup>[35]</sup>. However, the results from recent studies in India and elsewhere indicate that the severity of viral hepatitis during pregnancy is similar to that in non-pregnant women<sup>[36]</sup>.

Acute viral hepatitis in pregnancy requires differential diagnosis from liver disorders unique to pregnancy, in particular the HELLP syndrome, intrahepatic cholestasis of pregnancy (ICP), and acute fatty liver of pregnancy<sup>[37,38]</sup>. The HELLP syndrome is a variant of pre-eclampsia/eclampsia and is characterized by elevated liver enzymes, low platelet count and hemolysis. Its onset is generally in the second trimester of pregnancy, and patients may show initially elevated blood pressure, edema and proteinuria<sup>[39]</sup>. The intrahepatic cholestasis of pregnancy most frequently occurs in the third trimester of pregnancy with pruritus and elevated serum transaminases and bile salts; jaundice is rare and synthetic function of the liver is conserved<sup>[40]</sup>. Acute fatty liver of pregnancy is a severe condition that usually occurs in the third trimester with symptoms of encephalopathy and liver failure<sup>[41]</sup>. Early diagnosis is essential because treatment of acute hepatitis is generally conservative, whereas acute fatty liver of pregnancy and often HELLP syndrome require immediate delivery.

## CHRONIC HEPATITIS C

The most important studies of HCV in pregnancy have dealt with chronically infected women, and the natural

history of the liver disease in pregnant mothers and their offspring is not fully understood.

## EFFECT OF PREGNANCY ON HCV

During pregnancy in chronic HCV infection a significant reduction in the mean alanine aminotransferase (ALT) levels has been reported<sup>[4,42,43]</sup>, with rebound during the postpartum period. However, when we consider a cohort of pregnant women with HCV infection and persistently high aspartate aminotransferase/ALT levels, this trend is not confirmed<sup>[43]</sup>. The release of endogenous interferon from the placenta during pregnancy might partly explain changes in liver enzymes, but does not interfere with viral clearance<sup>[44]</sup>. Other factors, such as hemodilution or immune tolerance, may account for the decrease in serum transaminases during pregnancy. Sex hormones, and possibly immunosuppressive cytokines synthesized during pregnancy, might result in modulation of the immune response against HCV.

Observations regarding serum HCV-RNA concentration have been variable. Gervais *et al*<sup>[21]</sup> investigated a small number of pregnant women tested for viral load at regular intervals, and found that HCV RNA increased toward the end of pregnancy in some women. In our own study of 65 pregnant women tested during all three trimesters, we failed to show significant changes in viral load during and after pregnancy, although there was a trend toward an increase in the third trimester<sup>[43]</sup>. However, monitoring of viral load by monthly testing showed that HCV RNA is relatively stable over time in HCV chronic carriers without biochemical activity of the disease, whereas a low number of viremic flares can occur over a year in patients with biochemical activity of liver disease<sup>[45]</sup>.

Spontaneous clearance of HCV has been described in a single pregnant woman<sup>[46]</sup>. Only two reports describe significant worsening of histological disease consequent to pregnancy<sup>[47,48]</sup>. The study by Fontaine *et al*<sup>[48]</sup> included 12 cases with chronic hepatitis C and 12 controls without HCV infection. The first biopsy was done 1.6 years after delivery and the second at 4.3 years after the first biopsy. The overall Knodell score at initial biopsy was 4.8 in HCV-positive cases *vs* 5.3 in the controls. The Knodell score in the final biopsy was unchanged in controls, and was 8.4 in HCV-positive cases ( $P = 0.016$ ). The necroinflammation score showed 83% deterioration in cases and 25% in controls ( $P = 0.02$ ). The fibrosis score showed 41.6% deterioration in cases and 8.3% in controls ( $P > 0.05$ ). These findings in a small group of subjects, however, did not allow for any definite conclusion to be drawn.

### Effect of HCV on course of pregnancy

There is no unfavorable effect of HCV on pregnancy. In particular, three studies have addressed this question<sup>[18,49,50]</sup> (Table 3). The study from Jabeen *et al*<sup>[49]</sup> is particularly interesting, because it included a large cohort of rhesus-negative women in Ireland who became in-

**Table 3** Effect of hepatitis C virus on the course of pregnancy

	Hillemanns <i>et al</i> <sup>[19]</sup>	Jabeen <i>et al</i> <sup>[49]</sup>	Floreani <i>et al</i> <sup>[50]</sup>
Miscarriages	NA	12.4% vs 22.2%	-
Typical obstetric complications	-	-	-
Preterm delivery	29% vs 19%	4.5% vs 3.2%	NA
Rate of cesarean section	42% vs 21% ( <i>P</i> = 0.004)	5.6% vs 12.7%	41.50%
Fetal outcome	Good	Good	Good

NA: Not available.

fectured with HCV following exposure to contaminated anti-D immunoglobulin in 1977-1978. Thirty-six women who had been infected after their first pregnancy were compared to an age- and parity-matched control group of rhesus-positive women. Comparison with the control group showed no increase in spontaneous miscarriage rate, and no significant difference in obstetric complications. Taken together, these three studies have documented a good fetal outcome. The rate of cesarean section was significantly higher in the study by Hilleman *et al*<sup>[19]</sup> compared to controls (42% vs 21%, *P* = 0.004) and was similar to that observed in the Italian study<sup>[50]</sup>. The high rate of cesarean section in our study was due to the local protocol used in the past decade for reducing the rate of transmission of HCV in HCV-positive mothers, rather than peculiar obstetric indications for cesarean section.

In a population-based cohort study using Washington state birth records from 2003 to 2005, including 506 HCV-positive mothers, 2022 randomly selected HCV-negative mothers, and 1439 drug-using HCV-negative mothers, it was shown that infants born to HCV-positive women were more likely to have low birth weight, be small for gestational age, be admitted to the intensive care unit, or require assisted ventilation<sup>[51]</sup>.

In a more recent study using birth certificate records of 1670369 pregnancies, it was found that women with HCV were more likely to have infants born preterm, with low birth weight and congenital anomalies<sup>[52]</sup>. However, that study had several limitations, in particular, its retrospective design and the lack of association with several variables, such as use of tobacco, alcohol or drugs. Indeed, there is no explanation for prematurity and low birth weight in HCV-negative mothers, although increased cytotoxicity of placental natural killer T cells could be hypothesized possibility<sup>[53]</sup>.

It has also been reported that in pregnant women involved in a methadone treatment program, HCV reactivity was associated with an increased risk of neonatal withdrawal regardless of maternal methadone use<sup>[54]</sup>.

Risk factors for the development of ICP in HCV-positive mothers have been described. The first retrospective study reported a highly significant incidence of ICP in HCV-positive pregnant women compared with HCV-negative women<sup>[55]</sup>. Subsequently, another prospective Italian study confirmed these results and suggested the need to investigate the HCV status in women with ICP<sup>[56]</sup>.

In a study population of 21008 women with ICP identified from the Finnish Hospital Discharge Register during 1972-2000, hepatitis C was found to have a significantly higher incidence than in the controls<sup>[57]</sup>. More recently, a study of women with births between 1973 and 2009, registered in the Swedish Medical Birth Registry, confirmed a strong positive association between ICP and hepatitis C both before and after ICP diagnosis<sup>[58]</sup>. The link between ICP and HCV has not been completely explained so far, although several hypotheses can be suggested, including a defect in the transport of sulfated pregnancy hormones in the liver. It has been suggested that HCV downregulates the expression of the ABC transporter multi-drug resistance protein 2 (MRP2) in the liver, thus inducing failure in the transport of various toxic substances<sup>[59]</sup>. Furthermore, another link may be with a defect in the *ABCB11* gene encoding the bile salt export pump<sup>[60]</sup>.

### Vertical transmission of HCV

The overall rate of mother-to-child transmission of HCV from HCV-infected, HIV-negative mothers has been estimated at 3%-5%<sup>[61-66]</sup>. However, in an overview of 77 prospective cohort studies with at least 10 mother-infant pairs, the overall rate was 1.7% if the mother was known to be anti-HCV positive. If the mother was known to be viremic, that is HCV-RNA-positive, the rate was 4.3%<sup>[67]</sup>. At least one-third of infants acquire HCV infection during the intrauterine period; the perinatal transmission is estimated to be as high as 40%-50%, whereas postpartum transmission is rare<sup>[30,68]</sup>. The detection of HCV RNA in the serum of infants in the first 24 h of life suggests that early intrauterine infection may be possible<sup>[68]</sup>. The diagnosis of perinatal transmission should be considered in children born to HCV-positive mothers when: HCV RNA is detected in at least two serum samples at least 3 mo apart during the first year of life; and/or when testing of antibodies against HCV is positive after 18 mo of age<sup>[69]</sup>.

There is an interesting observation reported by the European Paediatric Hepatitis C Virus Network from a multicenter prospective study of HCV-infected pregnant women and their infants<sup>[69]</sup>. In that study girls were twice as likely to be infected as boys. This sex association is an intriguing finding that probably reflects biological differences in susceptibility or response to infection.

Co-infection with HIV increases the rate of mother-to-child transmission up to 19.4%<sup>[67]</sup>. The weighted rate of transmission is 8.6% in mothers who are anti-HCV positive and injecting drug users, compared with 3.4% in anti-HCV-positive mothers without known injecting drug use. A meta-analysis including 2382 infants estimated that the risk of HCV vertical transmission was 2.82 from anti-HCV<sup>+</sup>/HIV<sup>+</sup> co-infected mothers compared with anti-HCV<sup>+</sup>/HIV<sup>-</sup> mothers<sup>[70]</sup>. Vertical transmission of HIV and HCV separately is most likely from HIV/HCV-co-infected mothers; however, transmission of both infections is less frequent<sup>[71]</sup>.

Numerous risk factors for vertical transmission have been studied. In general, high viral load defined as at least

$2.5 \times 10^6$  viral RNA copies/mL, HIV co-infection, and invasive procedures are the most important factors<sup>[22]</sup>. In general, maternal peripheral blood mononuclear cell infection by HCV, membrane rupture > 6 h before delivery, and procedures exposing the infant to maternal blood infected with HCV during vaginal delivery are associated with an increased risk of transmission<sup>[69]</sup>. Abnormal ALT levels in mothers in the year before pregnancy may reflect a more severe liver disease and may help in identifying mothers with an increased risk of vertical transmission<sup>[72]</sup>. Finally, a Japanese study suggested that maternal liver dysfunction, large blood loss at delivery, and vaginal delivery were potential novel risk factors for mother-to-child transmission of HCV<sup>[66]</sup>.

## ANTIVIRAL THERAPY FOR CHRONIC HCV INFECTION

Antiviral therapy for HCV is contraindicated in pregnancy. Pegylated interferon would be problematic because of its psychiatric side effects in these women, who have a high background rate of postpartum depression<sup>[73]</sup>. Ribavirin carries the risk of teratogenicity for up to 7 mo after cessation of treatment. Treatment options should be offered before pregnancy in HCV-infected women. In fact, delay in initiation of antiviral therapy likely to be much longer than 9 mo, taking into account the postpartum recovery, breastfeeding and infant care. The benefits of considering treatment first and pregnancy second are superior to the drawbacks, including eliminating the risk of transmission of HCV to infants and reducing the risk of liver progression in mothers. Furthermore, improved efficacy of new drug regimens will require reassessment of the utility of universal screening for HCV in pregnant women<sup>[74]</sup>.

## MANAGEMENT OF HCV-INFECTED WOMEN

All women should receive antenatal screening for hepatitis B surface antigen and anti-HCV; HCV should be tested for in those found to be HCV positive, immigrants from developing countries, and in those with high-risk behavior (e.g., multiple sexual partners and intravenous drug use)<sup>[75]</sup>. A consensus for management of HCV-infected pregnant women and their children by the European Paediatric Network has been recently published<sup>[76]</sup>. The conclusions of this panel of experts indicate that although several risk factors for vertical transmission have been identified, none are modifiable and there are currently no interventions available to prevent such transmission. Based on the current evidence, it would be prudent to avoid amniocentesis, instrumented vaginal delivery, and prolonged rupture of membranes. A recent meta-analysis including 641 mother-infant pairs showed that cesarean section does not decrease perinatal HCV transmission from HCV-RNA<sup>+</sup>/HIV mothers to infants<sup>[77]</sup>. Thus elective cesarean delivery should not be offered, and breast-

feeding should not be discouraged. HCV/HIV co-infected women should be offered elective cesarean section to prevent HIV transmission and avoid breastfeeding where safe alternatives are available<sup>[78]</sup>.

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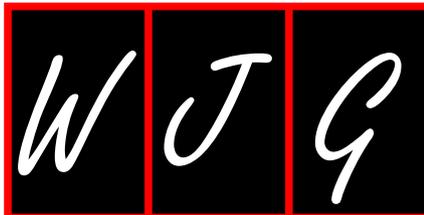
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WJG 20<sup>th</sup> Anniversary Special Issues (3): Inflammatory bowel disease

## Impact of exome sequencing in inflammatory bowel disease

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in advancing knowledge of the pathogenesis of IBD.

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**Core tip:** The genetic understanding of inflammatory bowel disease (IBD) has progressed over the last twenty years as new technologies and analytic techniques have become available. The nascent revolution in next-generation sequencing will enable us to sequence the exome - all the protein coding genes in the genome - in thousands of individuals. This review discusses the implications of this new approach for diagnosis in very early onset IBD and as a tool to gain understanding of the hereditary basis of the common polygenic form of the disease at the population level.

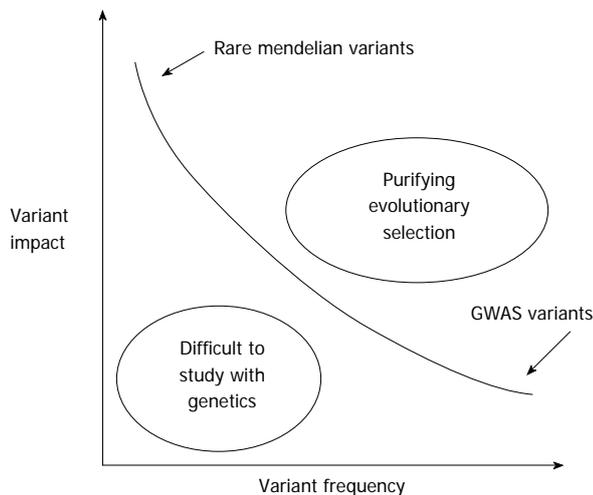
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### Abstract

Approaches to understanding the genetic contribution to inflammatory bowel disease (IBD) have continuously evolved from family- and population-based epidemiology, to linkage analysis, and most recently, to genome-wide association studies (GWAS). The next stage in this evolution seems to be the sequencing of the exome, that is, the regions of the human genome which encode proteins. The GWAS approach has been very fruitful in identifying at least 163 loci as being associated with IBD, and now, exome sequencing promises to take our genetic understanding to the next level. In this review we will discuss the possible contributions that can be made by an exome sequencing approach both at the individual patient level to aid with disease diagnosis and future therapies, as well as

### INTRODUCTION

The inflammatory bowel diseases (IBDs) consist of two main types of pathology: Crohn's disease and ulcerative colitis. Over the preceding decades, genetic epidemiology of twins and families indicated that these diseases have a strong genetic component, but that they do not segregate according to a Mendelian pattern of inheritance such as autosomal dominant, autosomal recessive, or X-linked<sup>[1]</sup>. Twin studies of Crohn's have shown a concordance of 20%-50% for monozygotic twins and 0%-7% for dizygotic twins<sup>[2]</sup>. For ulcerative colitis the concordance is 14%-19% for monozygotic and 0%-7% for dizygotic<sup>[2]</sup>. The fact that the monozygotic concor-



**Figure 1** Relationship between variant frequency and functional impact. Rare and highly damaging variants such as those associated with familial forms of very early onset inflammatory bowel disease (IBD) tend to occur rarely in the population. They are unable to achieve high allele frequencies due to negative evolutionary selection. Variants captured by genome-wide association studies (GWAS), and which account for much of the population attributable risk of IBD, tend to be quite common but have small functional consequences (typically OR < 1.2). Rare variants with small impacts are difficult to assess statistically with the tools of genetics. Exome sequencing is intended to fill in the middle part of the curve: less common variants that have moderate impacts.

dance is well below 100% shows that there are strong environmental contributions and that there is incomplete penetrance of the genetic susceptibility loci. At the same time, the risk is considerably elevated compared to the general population. Supported by the results of recent genome-wide association studies, the most commonly accepted model of IBD susceptibility is a multifactorial model in which polygenic inheritance at hundreds of genetic loci, each with small effects, contribute along with non-genetic factors, such as diet and microbiome composition.

One of the first successful approaches to identify specific risk genes was family-based linkage analysis. This approach seeks to identify chromosomal regions containing causative genes on the basis of recombinations within a family between a microsatellite marker and the trait of interest. Six loci were identified using linkage analysis, including the *IBD3* locus containing the human leukocyte antigen complex on chromosome 6, and the *IBD1* locus, the single largest genetic risk factor for Crohn's, which contains the nucleotide-binding oligomerization domain protein 2 (*NOD2*) gene on chromosome 16<sup>[3-5]</sup>.

The next technology to make a major impact in IBD genetics has been genome-wide association studies (GWAS). These studies involve genotyping hundreds of thousands of single nucleotide polymorphisms (SNPs) throughout the entire genome in order to find direct association between a specific polymorphism and the case/control status. The first successful study found an association between the interleukin (*IL*)23R locus and Crohn's disease in addition to replicating the *NOD2* as-

sociation<sup>[6]</sup>. Expanding the number of cases and controls in the cohort as genotyping prices dropped resulted in identification of *ATG16L1*<sup>[7]</sup>, *IRGM*, *MST1*, *NKX2-3*, and *PTPN2*<sup>[8,9]</sup>. The first IBD GWAS studies in a pediatric cohort were reported by our group, highlighting associations with *TNFRSF6B* and *IL27*<sup>[10,11]</sup>. As studies have grown more powered with increased cohort sizes, genotype imputation techniques<sup>[12]</sup>, and international collaboration through the IBD Genetics Consortium, the tally of associated loci for Crohn's and ulcerative colitis has risen to 163 in the latest meta-analysis<sup>[13]</sup>, demonstrating unequivocally the polygenic nature of IBD inheritance. Notably, the distribution of SNPs genotyped in a GWAS study covers intergenic as well as exonic and intronic regions, so that polymorphisms which predominantly affect the regulation of gene expression through transcriptional control can be assessed. Analysis of data from the ENCODE consortium has advanced the notion that much of the heritability of complex disorders originates in these non-coding regulatory regions of the genome<sup>[14]</sup>. There is no assumption in GWAS that the susceptibility or protective variants are confined to amino acid substitutions in proteins, the type of variation that would be found in exome sequencing. However, a major disadvantage of GWAS studies is that they are much more attuned to detecting common variation, that is, greater than 5% minor allele frequency for a SNP. It is worthy of note that IBD has generated a greater number of associations than any form of pathology studied genetically to date, leading some to suggest that evolutionary selective pressures for variants in the genes underlying the immune response drove autoimmune-risk alleles to relatively high frequencies, a phenomenon known as balancing selection<sup>[15]</sup>. The greater sensitivity of GWAS towards common variants is one reason among many that GWAS studies have only been able to account for a fraction of the heritability of polygenic diseases such as IBD<sup>[16]</sup>. It is becoming increasingly clear that there is more to the story than the common disease-common variant hypothesis<sup>[17]</sup>, and that rare variants, detectable only through sequencing, must also play a role<sup>[18,19]</sup>. Moreover, these coding variants are more likely to have high ORs, greater penetrance, and to be amenable to follow-up by functional experimentation<sup>[18]</sup>. Figure 1 illustrates the relationship between variant frequency and the phenotypic impact of the variant. Highly disruptive mutations will not rise to high frequency due to purifying selection. Exome sequencing is an ideal technology to fill in the intermediate frequency range of variants which may have stronger impacts than the weak associations detected by common GWAS variants.

## PROCESS OF EXOME SEQUENCING

With current technology, sequencing the whole 3 billion-base pair genome at the required depth of coverage to make rare variant calls is an expensive process, making it impractical to use on the scale required to implicate

less-common variants in IBD. Statistically validating less common variants with phenotypic impact would require GWAS-sized datasets comprising thousands of cases and controls<sup>[20]</sup>. A more practical alternative that has arisen since the emergence of next generation sequencing technology is to sequence the exome, the 1% of the genome that encodes protein. It has been estimated that 85% of monogenic, Mendelian disorders are the result of alterations in protein amino acid sequence<sup>[21]</sup>, supporting the idea that exon-focused sequencing will yield the most functionally interesting variants.

The most common way to fractionate the genome for exome sequencing is in-solution hybridization. This can be accomplished by shearing the DNA into small 200-300 bp fragments by ultrasonic or enzymatic methods followed by ligation of common adapter sequences to the 3' and 5' ends of the fragments so that the sequencing primer can anneal. This whole-genome library is captured by hybridization in solution with 50-120 nucleotide-long "baits" that are complementary to the exon sequence being targeted. The library-bound baits are bound to magnetic beads and the non-coding DNA is washed out. The captured fragments are eluted and amplified by PCR. Next generation sequencing instruments that utilize the exome library include the HiSeq and MiSeq (Illumina, Inc.) and Ion Torrent Proton (Life Technologies). The instruments sequence by synthesis with a DNA polymerase, analyzing the incorporation of the next nucleotide by fluorescence imaging with modified nucleotides (Illumina) or by electrical measurement of the protons produced by the incorporation of nucleotides (Ion Torrent). This generates a short "read" typically 100-200 bp in length, significantly shorter than the 700-bp reads produced by traditional Sanger sequencing. The reads are furnished as a list of sequences accompanied by quality metrics, known as a FASTQ file. One of these instruments can generate 20-60 gigabases of sequence per day.

The FASTQ file is analyzed by a read-mapping program, such as the popular Burrows-Wheeler aligner<sup>[22]</sup>, which matches these short reads with a reference genome. The alignment is stored in a common file format called BAM which is interpretable by a variety of analysis tools for visualization and variant identification. When enough independent reads have been aligned at the same nucleotide location in the genome, usually at least 20 reads, a variant calling application, for instance, Genome Analysis Toolkit<sup>[23,24]</sup>, the variant caller for the 1000 Genomes Project, can be used to decide if the site matches the reference sequence or contains an alternate nucleotide. The variant calls can be collected in a variety of formats, typically the Variant Call File (VCF). A range of statistical analyses can be performed on the VCF files for each exome, including annotating them for function (missense, indel, synonymous) and likely impact of the variant (damaging, tolerated) using tools such as ANNOVAR<sup>[25]</sup>, Sorting Intolerant From Tolerant<sup>[26,27]</sup>, and PolyPhen<sup>[28]</sup>. These tools use evolutionary

conservation of the gene across diverse species as well as the chemistry of the amino acid substitution to generate a predictive score of each variant's potential impact. The software tools also integrate information about the frequency of the variants in the general population using databases such as the The National Heart, Lung, and Blood Institute Exome Sequencing Project<sup>[29]</sup> and dbSNP<sup>[30]</sup>, since it is most likely that a damaging and impactful mutation would be quite rare due to purifying evolutionary selection.

In large case/control studies, the coding variants will be rarer than the polymorphisms identified through GWAS, so that any individual rare variant is unlikely to achieve a threshold of statistical significance<sup>[20]</sup>. Therefore, a variety of groups have developed methods to aggregate all of the rare variants in a gene and test them collectively in order to identify a rare variant burden in cases compared with controls or to detect an unusual distribution of variant frequencies between cases and controls for a given gene<sup>[31]</sup>. A number of these tests have the feature of being able to detect association in the presence of a mixture of risk, protective, and neutral variation<sup>[32]</sup>.

## ROLE FOR EXOME SEQUENCING IN IBD

### *Individual- and family-based sequencing for clinical use*

In attempting to identify a role for exome sequencing in inflammatory bowel disease we can appreciate two scenarios where it might be used. The first scenario is that of an individual patient or family with an atypical clinical presentation whose diagnosis or therapeutic decision may be influenced by genetic information. This can be seen in the very young children who present with clinical symptoms of IBD, known as very early onset IBD (VEO-IBD). These children frequently present with a more severe disease and often with a phenotype that is distinct from older children and adults, including extensive colonic disease unresponsive to standard therapy. These findings suggest distinct etiopathogenic pathways. In one well-known case, a 15-mo-old child presented with failure to thrive and perianal fistulae that was refractory to medical care. His disease progressed to pancolonic involvement, however the terminal ileum and upper tract were spared. This early age of onset and severity suggested a severe perturbation of the immune system<sup>[33]</sup>. He underwent numerous surgical procedures and treatment with immunosuppressive drugs, as well as targeted genetic and immunologic testing that did not yield a recognizable diagnosis or remission of symptoms. Sequencing of the child's exome revealed that this patient had an exceedingly rare mutation on the X chromosome in the *XIAP* gene, a potent regulator of the inflammatory response. He was treated by bone marrow transplant resulting in resolution of his disease. In our own IBD center at the Children's Hospital of Philadelphia, we encountered a 5-mo-old patient with colonic inflammatory bowel disease. She presented with severe disease that was unresponsive to medical therapy. Her

course was complicated by frequent episodes of dehydration and she became transfusion dependent despite various treatments. Exome sequencing in this patient revealed a mutation in the *MEFV* gene, resulting in a diagnosis of familial Mediterranean fever. The patient was referred to a pediatric rheumatology specialist and is being successfully treated for FMF with colchicine.

These successes highlight the critical role of exome sequencing in carefully selected patients by providing diagnoses that can guide treatment. Factors that suggest a patient may have a rare genetic perturbation that might be elucidated by exome sequencing would include early onset of disease, unusual severity, familial pattern of transmission, and a refractory response to standard therapies. In these cases, collecting DNA samples from parents so that exome sequencing in a trio setting can be performed is of high value. This will allow the identification of *de novo* variants as well as aiding in the elimination of the numerous false positive variant calls that exome sequencing generates by checking for non-Mendelian transmission of mutations. If a Mendelian inheritance model can be specified, as in the case of a consanguineous family which is likely to be autosomal recessive, such information can be of great help in narrowing down the causal variant. Homozygosity mapping in two consanguineous families was successfully used to identify mutations in the IL-10 receptor genes that resulted in severe VEO-IBD unresponsive to therapy. With this discovery, the disease resolved with bone marrow transplant<sup>[34]</sup>. This critical finding has been replicated in larger cohorts of patients with VEO-IBD and has shed light on an important pathway in the development of VEO-IBD<sup>[35]</sup>. A further appeal of applying exome sequencing in a family setting is in identifying novel monogenic causes of IBD that might yield an unexpected insight into the biology of disease, thereby directing interest towards novel targets for therapeutic development. An example would be the development of monoclonal antibodies that dramatically lower low-density lipoprotein (LDL) cholesterol by inhibiting proprotein convertase subtilisin kexin 9, a protein that was found to be deficient in a small number of individuals which genetically very low LDL<sup>[36]</sup>.

### Somatic mutations

An area where exome sequencing has been impactful is in the sequencing of cancer tissue exomes in comparison with the patient's inherited exome. Some studies have been successful in identifying somatic "driver mutations" which are essential for the growth of the tumor, which can spur the development of chemotherapeutic interventions that will target the cancer specifically<sup>[37,38]</sup>. Great interest has sprung up around the promise of personalized, or precision, medicine for cancer driven by the somatic genomics of tumors<sup>[39]</sup>. Whether sequencing of intestinal biopsies in IBD present an avenue to identify somatic mutations that may be critically important for microbiome interaction is yet to be determined, but studies are underway that are addressing this possibility.

### Exome sequencing as a research tool to complement GWAS

The second scenario in which exome sequencing can be impactful is as a research tool to augment GWAS in uncovering novel susceptibility loci and specific coding variants in the typical polygenic form of Crohn's and ulcerative colitis. Whether exome sequencing will succeed in this role to the same degree as GWAS is still controversial. It is clear that identifying genes carrying a burden of exonic rare variants in a disease with the highly polygenic architecture of IBD will require GWAS-sized cohorts, that is, ones consisting of tens of thousands of cases and controls<sup>[40]</sup>. The high cost and labor intensity of such an effort currently makes these studies prohibitively expensive to all but the most resource-rich groups. Nevertheless, some groups have succeeded in finding rare variant associations through sequencing at the phenotypic extremes of several complex traits in carefully selected candidate genes such as *ANGPTL4* and *ANGPTL5*<sup>[41]</sup> or *LPL*<sup>[42]</sup> in triglycerides, *SLC12A1* in blood pressure<sup>[43]</sup>, and *IFIH1* in type 1 diabetes<sup>[44]</sup>. Targeted next-generation sequencing in IBD has even produced some rare variant associations by following up GWAS hits, such as coding mutations that reduce signaling through the IL-23 receptor<sup>[45]</sup>. Targeted next-generation sequencing by Rivas *et al.*<sup>[46]</sup> identified additional *NOD2* and *IL23R* coding variants, as well as novel coding variants in *CARD9*, *IL18RAP*, *CUL2*, *C1orf106*, *PTPN22* and *MUC19*. Our group has also recently identified rare nonsynonymous variants in the *TNFRSF6B* gene in IBD patients with pediatric onset disease<sup>[47]</sup>, suggesting that this could be true for other GWAS loci as well. Table 1 summarizes genes that have been shown to have nonsynonymous variants with disease relevance in IBD.

Despite the success of these candidate gene efforts, doubts remain about how practical rare variant studies will be when applied to the entire exome. Most of the rare variant associations identified so far in candidate gene sequencing would not meet the stringent Bonferroni correction for multiple testing on an exome scale, estimated to be a  $P < 2.5 \times 10^{-6}$ <sup>[31]</sup>. Investigators must also consider that supporting novel rare variant associations requires replication in additional cohorts since rare variants are often population-specific and frequencies can vary in very inhomogeneous ways in spatially structured populations<sup>[48]</sup>. This type of confounding, known as population stratification, can lead to spurious associations. Therefore, replication would likely require additional sequencing of large cohorts since genotyping of specific variants would likely not be useful in a different geography or ethnicity<sup>[49]</sup>, although the replication sequencing might be limited only to genes of interest in the discovery cohort.

Concerns about the likelihood of uncovering a substantial amount of heritability in common autoimmune diseases was raised by a recent report by Hunt *et al.*<sup>[50]</sup>. This effort selected 25 risk genes that were identified in GWAS of at least two different common autoimmune

**Table 1 Genes implicated as having coding variants in inflammatory bowel disease**

Gene symbol	Description	Ref.
ADAM17	Disintegrin and metalloproteinase domain-containing protein 17	[57]
ATG16L1	Autophagy related 16-like 1	[7]
C1orf106	Chromosome 1 open reading frame 106	[46]
CARD9	Caspase recruitment domain family, member 9	[46]
CUL2	Cullin 2	[46]
DLG5	Discs-large 5	[58]
HEATR3	HEAT repeat containing 3	[59]
IL10RA and B	Interleukin-10 receptor	[34]
IL18RAP	Interleukin 18 receptor accessory protein	[46]
IL23R	Interleukin 23 receptor	[45]
LRBA	LPS-responsive vesicle trafficking, beach and anchor containing	[60]
MDR1	Multi-drug resistance 1	[61]
MUC19	Mucin 19, oligomeric	
NCF2	Neutrophil cytosolic factor 2	[62]
NDP52	Antigen nuclear dot 52 kDa Protein	[55]
NOD2	Nucleotide-binding oligomerization domain containing 2	[3,4]
PRDM1	PR domain containing 1, with Zinc finger domain	[55]
PTN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	[46]
SLC22A4	Solute carrier family 22 (organic cation/ergothioneine transporter), member 4	[63]
TNFRSF6B	Tumor necrosis factor receptor superfamily, member 6b, decoy	[47]

diseases. The exons of these 25 genes were sequenced with excellent coverage in a cohort of 24892 subjects with six autoimmune disease phenotypes and 17019 controls. They found that the great majority of variants uncovered occurred in a single subject. Five aggregating gene-based tests (rather than individual variant-based tests) were used to identify rare-variant enrichment for any of the genes but none were found to be statistically significant. The authors concluded that there was little support for large-scale whole-exome sequencing projects in common autoimmune diseases. While this report may portend that the impact from rare coding variants is negligible, there are some limitations to the study that leave open the possibility for meaningful discovery. The study considered only 25 genes, while an exome-based approach would survey all 20000 human protein-coding genes. It is likely that the risk conferred by these 25 GWAS genes is carried by non-coding variation, while the risk at some subset of loci in the genome could be carried by rare coding variation that are not captured by GWAS SNPs. This is particularly true for variants in the intermediate 0.5%-5% frequency range which could be impactful in aggregate while escaping detection in GWAS studies due to the weak linkage disequilibrium for variants in this frequency range with common variants<sup>[51]</sup>. Indeed, Hunt *et al*<sup>[50]</sup> did identify three risk mutations at approximately the 5% minor allele frequency. Furthermore, the abundant singleton mutations could still identify IBD risk genes through the use of statisti-

cal tests which weight mutations more or less heavily depending on their frequency, as the very rare variants are the most likely to be impactful functionally. Several methods such as adaptive sum tests<sup>[52]</sup>, Sequence Kernel Association Test<sup>[53]</sup>, and Variable Threshold<sup>[54]</sup> tests have been developed specifically for sustaining a high statistical power with rare variants. Finally, the six autoimmune disease phenotypes were quite heterogeneous in their pathologic nature, ranging from IBD to autoimmune thyroid disease to multiple sclerosis. These diseases have distinct mechanisms with different rare variants underlying them, possibly in none of the 25 genes sequenced. Therefore, it is arguably diluting the power to detect rare variant association by combining diverse diseases.

A recent study by Ellinghaus *et al*<sup>[55]</sup> utilized exome sequencing to identify a role for missense variants in *PRDM1* and *NDP52* in Crohn's disease. Variants in these two genes were discovered in a cohort of 42 whole-exome sequenced individuals, with discovered variants being prioritized by functional impact scores and presence within GWAS-delineated loci. Over 20000 combined Crohn's and ulcerative colitis cases and controls were genotyped to establish that two variants, p.Ser354Asn in *PRDM1* and p.Val248Ala in *NDP52* were associated with IBD. Functional studies showed that the *PRDM1* mutant increased T cell proliferation and cytokine secretion while the *NDP52* mutant impaired the ability of the protein to downregulate nuclear factor kappa B signaling in toll-like receptor signaling pathways. This paper provides an example of how exome sequencing, even in a modest cohort, can refine GWAS signals and uncover less common risk variants, especially when coupled with functional validation.

### Sequencing and risk prediction

Our group recently developed a machine-learning approach to predicting risk for IBD using data from the International IBD Genetics Consortium's ImmunoChip project<sup>[56]</sup>. The ImmunoChip assays 200000 SNPs with very dense coverage in genomic regions that have been associated with autoimmune disease through genome-wide association studies. Due to the ImmunoChip's wide spectrum of variants and the large number of cases and controls genotyped in the project, it was possible to use a penalized logistic regression model to predict risk for IBD with area under the curve of 0.86 for Crohn's disease and 0.83 for ulcerative colitis. With the coming availability of large-scale whole exome data we expect that risk prediction can be improved further and may achieve clinically-useful levels with the comprehensive catalog of variation that would be produced through eventual whole-genome sequencing.

## CONCLUSION

We can predict with some confidence that exome sequencing will have a place in IBD in a patient- or family-based settings where features of the clinical presentation

suggest a likely monogenic, Mendelian basis for the disease. Personalized medicine based on the patient's genome in these carefully selected cases is no longer a far-off dream but a nascent reality. More uncertain are the prospects of large-scale exome sequencing projects for discovery of population-scale heritability for such a common and highly polygenic disease. Theoretical arguments can be made to support either position, but the debate can only be resolved by experimental testing of the common disease-rare variant hypothesis. Exome sequencing of rare variants may not collectively yield much explanation of the population attributable risk of disease, but it has great potential to highlight the key players in the pathogenesis of disease along with variants amenable to functional study and thereby influence the development of potent new therapeutics.

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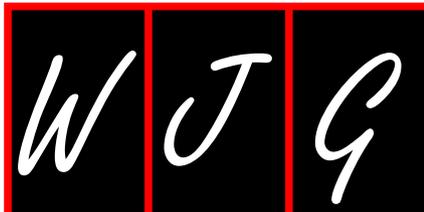
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## Virus entry mediated by hepatitis B virus envelope proteins

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### Abstract

Hepatitis B virus (HBV), a major cause of human liver disease worldwide, encodes three envelope proteins needed for the attachment and entry of the virus into susceptible host cells. A second virus, hepatitis delta virus, which is known to enhance liver disease in HBV infected patients, diverts the same HBV envelope proteins to achieve its own assembly and infection. In the lab, lentiviral vectors based on human immunodeficiency virus type 1 can be assembled using the HBV envelope proteins, and will similarly infect susceptible cells. This article provides a partial review and some personal reflections of how these three viruses infect and of how recipient cells become susceptible, along with some consideration of questions that remain to be answered.

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**Key words:** Hepatitis B virus; Hepatitis delta virus; Receptor; Envelope proteins; Entry

**Core tip:** The recent identification of a key receptor for hepatitis B virus and hepatitis delta virus provokes a wider discussion of how different cells may become susceptible to infection when the receptor is provided.

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### INTRODUCTION

The following is a very brief introduction to the envelope proteins of hepatitis B virus (HBV) and of how they can be used to facilitate the assembly and infection by HBV and two other viruses. More detailed recent reviews are available elsewhere<sup>[1,2]</sup>. HBV encodes three envelope proteins, commonly referred *via* their size, as large, middle and small, or L, M and S. They have S as a common C-terminal domain. M contains additional N-terminal sequences, referred to as preS2. L, relative to M, has additional N-terminal sequences, referred to as preS1. L, M and S, exist with and without carbohydrate modifications. The L protein undergoes an essential myristoylation of a glycine residue penultimate to the N-terminus.

Hepatitis delta virus (HDV) exists in nature in some patients who are also infected with HBV. While HDV uses a totally different method of genome replication than HBV, its final assembly is entirely dependent upon the envelope proteins of HBV; thus, it only produces infectious progeny in hepatocytes already infected with HBV (or producing envelope proteins from fortuitously integrated HBV DNA). HDV can infect a new hepatocyte in the presence or absence of HBV. In the humanized chimeric uPA mouse model, human hepatocytes infected with HDV alone can persist for at least six weeks in the absence of HBV, a so-called latent infection, with ultimate rescue of virus production dependent on a follow-up infection by HBV<sup>[3]</sup>. Such studies suggest that in patients conversion of a latent HDV mono-infection may contribute to the persistence of HDV even in patients with low HBV replication.

Many labs have produced retrovirus vectors that have

been engineered to carry novel genes, that can be expressed following integration of the provirus; that is, the DNA copy of the viral RNA genome. Such retrovirus vectors have been assembled using envelope proteins of the wild type retrovirus, as well as those derived from other viruses, such as vesicular stomatitis or Ebola viruses<sup>[4]</sup>. However, what is relevant here is that such a retrovirus vector was assembled using the envelope proteins of HBV and acquired the host cell specificity of HBV and HDV<sup>[5]</sup>.

## ENVELOPE PROTEIN DETERMINANTS ESSENTIAL FOR INFECTIVITY

Several labs have shown that an essential determinant for infectivity using HBV envelope proteins is located near the N-terminus of the preS1 domain of the L protein. The data for this are very good and include evidence that a synthetic peptide containing such sequences, especially if it is myristoylated, will act as a potent inhibitor of virus entry<sup>[6]</sup>. Interestingly, unlike the L and S proteins, the M protein can be omitted in experimental situations without a loss of assembly or infectivity, at least for HDV<sup>[7,8]</sup>. Thus, the role of M in HBV infection is unclear. The carbohydrate moieties attached to the three envelope proteins are essential for particle assembly and infectivity<sup>[2]</sup>. The three envelope proteins share at least four trans-membrane domains. One shared loop is presented on the surface of the virus. This loop, containing the so-called "A" determinant, is highly antigenic. Certainly antibodies raised against the S protein will neutralize virus infectivity. A widely used, S protein based vaccine, protects individuals against both HBV and HDV.

## WHAT THE HOSTS PROVIDE

Until last year, almost the only cells that could be infected by these viruses were primary human hepatocyte cultures. Such cultures are difficult both to establish, and can rapidly lose susceptibility to infection. Some non-human primary hepatocyte cultures were similarly susceptible; examples include chimpanzee and tupaia hepatocytes. Also, primary woodchuck hepatocytes are susceptible to HDV. Several years ago a cell line, HepaRG, was derived from a human liver tumor, and it is susceptible to infection by HBV and HDV<sup>[9]</sup>. These cells require specific *in vitro* culture conditions and they are almost as difficult to maintain as primary human hepatocytes.

For many years groups worldwide had struggled to identify, and confirm the functionality of host molecules needed for HBV and HDV entry. Many candidates were identified but none were shown to be sufficient for virus entry and initiation of replication<sup>[6]</sup>. This situation was changed dramatically in late 2012 by a report from Yan *et al.*<sup>[10]</sup>. They used a synthetic peptide corresponding to the myristoylated N-terminus of the HBV preS1 protein to affinity select a candidate virus receptor from hepatocyte cultures. These hepatocytes were derived the treeshrew

(*Tupaia belangeri*). The purification procedure used near-zero-distance photo-cross-linking and tandem-affinity purification and yielded a single protein. Then, with mass spectrometry, they identified the protein as the sodium taurocholate cotransporting polypeptide, NTCP, also known as SLC10A1<sup>[10]</sup>. NTCP transports bile acids from the blood into the liver. Their subsequent findings included evidence that the cDNA clone of human NTCP, when transfected into human hepatocellular carcinoma cell lines, specifically HepG2 and Huh7, conferred susceptibility to both HBV and HDV. Susceptibility could be inhibited by the synthetic preS1 peptide. Furthermore, in susceptible primary hepatocyte cultures and HepaRG cells, suppression of NTCP with specific small interfering RNAs inhibited susceptibility. It remains to be shown whether the NTCP functions *in vivo* as well as *in vitro*. However, the situation is very promising in that other studies have already shown that the synthetic preS1 peptide to which it binds, is a potent inhibitor of *in vivo* infections of human hepatocytes (as transplanted into mice) by both HBV and HDV<sup>[11]</sup>.

In their initial paper, the authors made use of prior studies by others of the established role of NTCP in the liver. This protein is 335 amino acids in length and is predicted to have 9 trans-membrane domains<sup>[12]</sup>. They thus compared the sequence of NTCP of the crab-eating monkey (*Macaca fascicularis*) (since hepatocytes from these monkeys are not susceptible to HBV infection) and humans. They noted that the primary sequence of the monkey protein has a limited number of specific differences relative to the human protein. The authors experimentally demonstrated that replacement of just nine contiguous amino acids of monkey NTCP with the corresponding sequence from the human protein produced a cDNA, that when transfected into nonsusceptible liver cell lines, rendered them susceptible to infection by both HBV and HDV.

Several commentaries by others on the NTCP findings have since been published<sup>[13-16]</sup>. And, several labs, in as yet unpublished studies, have confirmed the ability of recombinant NTCP to facilitate entry of HBV and HDV into otherwise non-susceptible cell lines.

Moreover, two follow-up studies by Wenhui Li and coworkers have been published in 2013. In the first study, the focus was on the woolly monkey hepadnavirus, WMHBV, and its ability to infect tupaia hepatocytes<sup>[17]</sup>. Their findings include evidence that cDNA of the woolly monkey NTCP when transfected into nonsusceptible liver cell lines renders them susceptible to both WMHBV and HDV pseudotyped with WMHBV envelope proteins. The authors thus suggest that orthologs of NTCP might function as receptors for all known primate hepadnaviruses. In the second study they examined mouse NTCP, and considered many exchanges with the human sequence to determine what might be need to achieve susceptibility<sup>[18]</sup>. They did find a region, of only two amino acids, that was sufficient to achieve HDV (but, as discussed below, not HBV) susceptibility. These changes

were not at the same location as the changes needed on the crab-eating monkey NTCP<sup>[10]</sup>. We can infer that the secondary and maybe the tertiary structure of NTCP are needed for susceptibility. It is relevant that a recently published study by Meier *et al*<sup>[19]</sup> reports that cultured mouse hepatocytes will bind the preS1 peptide even though no infection is detected. This suggests that a *bona fide* binding site may be available on the mouse NTCP, but is insufficient for HBV infection. Scheick *et al*<sup>[20]</sup> suggest an additional step might be at the level of membrane fusion.

It is important to note that earlier studies showed that HDV can actually infect mouse hepatocytes *in vivo*, although to only a low extent<sup>[21]</sup>. Therefore, the changes introduced into the mouse NTCP, as described above, may have increased the affinity of preS1 binding without, for HDV, a need to alter a second step (*e.g.*, fusion) in HDV infection. Thus, it is possible that mice made transgenic for the modified NTCP will be readily infected with HDV. (They will not be infected by HBV and as such, may not be sufficient to create a new model of HBV induced hepatocellular carcinoma.) Or, perhaps, even overexpression of the unmodified mouse NTCP would allow more efficient *in vivo* infection by HDV.

## ATTACHMENT AND ENTRY

Recent studies have shown that the initial attachment of HBV to susceptible cells depends upon glycosaminoglycans present on the cell surface<sup>[22-24]</sup>. This is a necessary but not sufficient step. Furthermore, even after virus has attached, entry can be significantly inhibited by a number of agents<sup>[25]</sup>. Of the latter, the most interesting inhibitor is the previously mentioned synthetic peptide, corresponding to the N-terminus of the preS1 domain<sup>[6,11]</sup>. It would seem that even after attachment, (additional) interactions with the putative host cell receptor remain to be made<sup>[25]</sup>. It could also be that multiple such interactions are needed to facilitate entry.

## FURTHER DISCUSSION AND OUTLOOK

Clearly, the combination of attachment, entry, and initiation of HBV replication is more complex than that of HDV. A partial explanation of this difference comes from earlier studies: Firstly, transcription of RNA from HBV covalently closed-circular DNA (CCC DNA) depends upon host transcription factors, some of which are specific for the liver<sup>[2]</sup>. Additionally, CCC DNA formation is inefficient or even absent in transgenic mice<sup>[26]</sup>. Secondly, HDV replication can be initiated in many mammalian cell lines, not necessarily those that are derived from liver tissue<sup>[1]</sup>. Nevertheless, it also remains possible that it is the entry of HBV that differs from that of HDV; that is, it requires host contributions in addition to the expression of NTCP.

As exemplified below, there are now multiple questions that can be asked of the entry mechanisms of other members of the hepadnavirus family. Certainly it is time

to reinvestigate infection of duck hepatocytes by duck hepatitis B virus (DHBV). It was first found by an affinity strategy, that a host protein identified as carboxy peptidase D, binds the DHBV envelope protein<sup>[27]</sup>. However, no study has yet been able to confer susceptibility by expression of this protein<sup>[28,29]</sup>. Some studies have suggested a co-receptor, but again, this has not led to susceptibility<sup>[30]</sup>. Independently, it has been shown that the myristoylated N-terminus of the DHBV preS region is needed for infectivity<sup>[28,31]</sup>. Therefore, maybe an analogous application of the powerful peptide affinity strategy used by Wenhui Li and coworkers<sup>[10]</sup>, will identify a functional DHBV receptor. And, an alternative and specific approach would be to directly test whether the duck NTCP can function as receptor.

Also one can examine the newly reported HBV of bats<sup>[32]</sup>. This virus shows more sequence relationship to the orthohepadnavirus than to the avi hepadnaviruses. Novel questions can now be asked, such as what is the sequence of the bat NTCP, and would the cDNA of this gene when expressed in human cell lines, confer susceptibility to infection.

Another question is why HDV but not HBV will infect primary woodchuck hepatocytes. This may or may not be analogous to the abovementioned situation where human NTCP cDNA transfected into non-liver cell lines will allow HDV but not HBV infection. Presumably the woodchuck hepatocytes provides an acceptable NTCP for HDV infection, but now one can address questions such as whether expression of one or more human cDNAs will make the cells susceptible to HBV.

In all these studies of cells susceptible to HDV or HBV, there is a question of the efficiency with which cells are infected. Even with primary hepatocytes and HepaRG cell line, the efficiency is typically less than 1%. Several possible explanations include low levels of expression of NTCP or low levels of NTCP that is functionally active. This efficiency is increased approximately 10-fold by the presence of 2%-4% polyethylene glycol, PEG<sup>[9,33]</sup>. Studies have shown that infection in the presence of PEG is still be inhibited by the synthetic preS1 peptide, indicating that the infection is still specific for the NTCP receptor. PEG is most likely causing aggregates of virus particles; certainly, at 6%-10% PEG the virus can even be collected by low speed centrifugation. Thus, it may be that the low levels of PEG produce aggregates that can more efficiently make use of the available NTCP receptor present on the surface of the hepatocytes. Future studies with cDNA transfected cell lines will no doubt test the relevance of the amount of NTCP expressed at the cell surface and infection by unaggregated *vs* aggregated virus. Perhaps controlled mixtures of functional and non-functional NTCP cDNAs will also help clarify what is involved.

In summary, the important new finding of NTCP as a functional receptor has opened the way for much more basic research concerning the entry of HBV and HDV into susceptible cells. And, in turn, such information will

allow new applied research, possibly providing additional novel ways in which such entry can be interfered with, all new armamentaria for treating chronic HBV and HDV infections.

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## Pathogenesis of hepatic steatosis: The link between hypercortisolism and non-alcoholic fatty liver disease

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### Abstract

Based on the available literature, non alcoholic fatty liver disease or generally speaking, hepatic steatosis, is more frequent among people with diabetes and obesity, and is almost universally present amongst morbidly obese diabetic patients. Non alcoholic fatty liver disease is being increasingly recognized as a common liver condition in the developed world, with non alcoholic steatohepatitis projected to be the leading cause of liver transplantation. Previous data report that only 20% of patients with Cushing's syndrome have hepatic steatosis. Aiming at clarifying the reasons whereby patients suffering from Cushing's syndrome - a condition characterized by profound metabolic changes - present low prevalence of hepatic steatosis, the Authors reviewed the current concepts on the link between hypercortisolism and obesity/metabolic syndrome. They hypothesize that this low prevalence of fat accumulation in the liver of patients with Cushing's syndrome could result from the inhibition of the so-called low-grade chronic-

inflammation, mainly mediated by Interleukin 6, due to an excess of cortisol, a hormone characterized by an anti-inflammatory effect. The Cushing's syndrome, speculatively considered as an *in vivo* model of the hepatic steatosis, could also help clarify the mechanisms of non alcoholic fatty liver disease.

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**Key words:** Nonalcoholic fatty liver disease; Cushing's syndrome; Hypercortisolism

**Core tip:** This overview of the literature is related to hepatic steatosis, its prevalence, clinical consequences and, in particular, the pathogenesis of this disorder. The authors focus on the link between hypercortisolism and obesity/metabolic syndrome. The main question of the work relates to the low prevalence of hepatic steatosis (only 20%) described in 50 newly diagnosed patients with Cushing's syndrome based on appropriate computed tomography scans available for retrospective analysis. The authors try to explain this finding by the anti-inflammatory effect of high circulating levels of glucocorticoids.

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### INTRODUCTION

In Cushing's syndrome, high circulating glucocorticoid (GC) levels cause visceral obesity, insulin resistance, diabetes mellitus, dyslipidemia, hypertension, hepatic steatosis and an increased risk of coronary artery disease (CAD)<sup>[1,2]</sup>. GCs excess stimulates gluconeogenesis in the

liver and inhibits insulin sensitivity both in the liver and in skeletal muscles, which represent the most important sites responsible for glucose metabolism. In particular, glucocorticoid excess stimulates the expression of several key enzymes involved in the process of gluconeogenesis, with a consequent increase in glucose production, and an impairment of insulin sensitivity either directly by interfering with the insulin receptor signaling pathway or indirectly, through the stimulation of lipolysis and proteolysis and the consequent increase in fatty acids and amino acids, which contribute to the development of insulin resistance (IR). Moreover, the peculiar distribution of adipose tissue throughout the body, with the predominance of visceral adipose tissue, significantly contributes to the worsening of IR and to the development of a metabolic syndrome, which has a role in the onset and maintenance of impaired glucose tolerance<sup>[3]</sup>.

## METABOLIC SYNDROME AND 11 $\beta$ -HYDROXYSTEROID DEHYDROGENASE TYPE 1

The much more prevalent “metabolic syndrome” a medical condition with a clustering of risk factors for cardiovascular disease and type 2 diabetes, such as IR, type 2 diabetes, dyslipidemia and hypertension, typically in association with visceral obesity and hepatic steatosis shares metabolic alterations and physical findings with Cushing’s syndrome<sup>[1]</sup>. However, any pathogenic role for GCs in the metabolic syndrome or idiopathic obesity is still unclear<sup>[4]</sup>. Recent studies in humans and rodents suggest a role for tissue rather than plasma GCs excess in the development of idiopathic obesity and the metabolic syndrome, *via* intracellular steroid reactivation of inert circulating 11-dehydrocorticosterone (cortisone in humans) into active corticosterone (cortisol) by 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) type 1. Surprisingly, this enzyme is highly expressed not only in adipose tissue and brain but in the liver as well<sup>[5]</sup>.

The 2- to 3-fold-increased 11 $\beta$ -HSD1 activity in adipose tissue in obese Zucker rats<sup>[6]</sup> and in some<sup>[7,8]</sup> but not all<sup>[9]</sup> studies on obese humans may be causal to visceral obesity and its metabolic consequences. Supporting this hypothesis, visceral obesity, hyperlipidemia, IR, glucose intolerance/diabetes<sup>[10]</sup> and hypertension<sup>[11]</sup> are driven in transgenic mice by overexpression (2- to 3-fold) of 11 $\beta$ -HSD1 selectively in adipose tissue.

Notably, as with the human metabolic syndrome, circulating plasma corticosterone levels in aP2-HSD1 TG mice are unaltered<sup>[10]</sup>. Conversely, 11 $\beta$ -HSD1 null mice exhibit a protective glycemic, lipid, and lipoprotein profile<sup>[12,13]</sup> and show increased expression of hepatic mRNAs encoding regulators of fatty acid beta-oxidation<sup>[13]</sup>. While intra-adipose but not systemic corticosterone concentrations are elevated in aP2-HSD1 TG mice, corticosterone delivery to the liver is also increased about three-fold *via* spillover of adipose steroid production into

the portal vein. The highest expression of 11 $\beta$ -HSD1 occurs in the liver<sup>[14]</sup>, and hepatic 11 $\beta$ -HSD1 mRNA levels are regulated by diet, gender and hormones<sup>[1,14-16]</sup>. Heterogeneity of hepatic 11 $\beta$ -HSD1 activity may be relevant to the development of specific fatty liver, insulin-resistant, and hypertensive syndromes without obesity in humans; it is also likely to play a role in myotonic dystrophy, where marked insulin resistance and dyslipidemia have been shown to occur with elevated hepatic 11 $\beta$ -reduction of cortisone to cortisol, which is positively correlated to the severity of disease<sup>[17]</sup>.

Dysregulation of the specific action of GCs and not of the alterations of GC levels has been proposed as a central feature of the metabolic syndrome<sup>[18]</sup>. In fact, states of GC excess recapitulate almost all features of the metabolic syndrome, but Cushing’s disease is rare and circulating cortisol levels are normal in the vast majority of patients with obesity and type 2 diabetes. This finding has raised the possibility that the features of the metabolic syndrome could be due to an increase in locally available glucocorticoids through 11 $\beta$ -HSD1<sup>[19-21]</sup>. Subsequently, a range of studies explored the role of 11 $\beta$ -HSD1 in the pathogenesis of the components of the metabolic syndrome including obesity, IR, hyperglycemia, hyperlipidemia and nonalcoholic fatty liver disease (NAFLD), or generally speaking, hepatic steatosis.

### *Hepatic steatosis and visceral fat*

Indeed, only 20% of patients with Cushing’s syndrome have hepatic steatosis<sup>[22]</sup>. NAFLD ranges from fatty liver to non alcoholic steatohepatitis (NASH) and cirrhosis, and is being increasingly recognized as the most common liver disease in the developed world. NASH - which most Authors consider as completely different from the more benign form, *i.e.*, fatty liver - is projected to be the leading cause of liver transplantation. NAFLD has a great prevalence among people with diabetes and obesity and is almost universally present amongst morbidly obese diabetic patients<sup>[23-25]</sup>.

Being a progressive form of liver injury, NASH carries a risk of developing hepatocarcinoma. There is strong evidence that IR and increased free fatty acids are the major cause (“first hit”) of NASH<sup>[26,27]</sup>. Inflammation plays an important additional role (“second hit”) with increased production of reactive oxygen species and proinflammatory cytokines. In addition, studies from different groups support the strict link between visceral adipose tissue and NASH<sup>[28,29]</sup>.

In recent years, research has shown that amplification of GCs action by the intracellular enzyme 11 $\beta$ -HSD1 plays a key role in the development of central obesity<sup>[30,31]</sup> providing a basis for the phenotypic similarity between Cushing’s syndrome and obesity in the metabolic syndrome; 11 $\beta$ -HSD1 increases intracellular glucocorticoid levels by converting inert cortisone to active cortisol.

*In vivo* reductase activity of 11 $\beta$ -HSD1 predominates and is driven by NADPH generated by the microsomal enzyme hexose-6-phosphate-dehydrogenase (H6PDH).

Epidemiological data indicate that hepatic steatosis is associated with IR, dyslipidemia and obesity, especially central obesity<sup>[32]</sup>. In clinical practice, the co-existence of these conditions defines the so-called metabolic syndrome<sup>[33]</sup>. Of note, NAFLD is considered by many authors as the hepatic manifestation of the metabolic syndrome<sup>[34-36]</sup>.

Interestingly, the severity of hepatic steatosis is positively correlated with visceral adipose tissue accumulation in both obese and non-obese subjects, suggesting that hepatic fat infiltration may be influenced by visceral fat adipokines or, possibly, specific enzymes, regardless of body mass index<sup>[37]</sup>.

As previously emphasized, some authors have pointed out the phenotypic similarities between central obesity, metabolic syndrome and patients with endogenous or exogenous glucocorticoid excess. These have led them to propose that cortisol contributes, at least in part, to the pathogenesis of those abnormalities, despite the fact that patients with obesity and metabolic syndrome have consistently normal cortisol levels in plasma and urine<sup>[38-40]</sup>. Accordingly, the concentrations of GCs found in the omental vein, draining into the portal vein, were not different from those detected in peripheral veins<sup>[41]</sup>.

A plausible explanation for this phenomenon could be to consider the metabolic syndrome a result of increased local GC activity in certain organs, suggesting that central obesity might be, as proposed by Bujalska decades ago, a “Cushing’s disease of the omentum”<sup>[42]</sup>. In connection with this concept, recent studies have shown that intracellular GC action not only depends upon the hypothalamo-pituitary-adrenal axis but also on local regulation at the pre-receptor level by the activity of two isoforms of the 11 $\beta$ -hydroxysteroid dehydrogenase enzyme type 1 and 2 (11 $\beta$ -HSD1 and 11 $\beta$ -HSD2)<sup>[43,44]</sup>.

### 11 $\beta$ -hydroxysteroid dehydrogenase type 1 and visceral adipose tissue

Enzyme type 1, 11 $\beta$ -HSD1, is a microsomal enzyme, expressed mainly in the liver and adipose tissue, acting as a NADP (H)-dependent reductase converting inactive cortisone to active cortisol, which locally activates GCs receptors<sup>[45]</sup>. According to this view, progressive expansion of visceral fat would result in an increased production of cortisol by the action of 11 $\beta$ -HSD1, causing splanchnic and portal hypercortisolism, which could contribute to the pathogenesis of such metabolic disorders, including NAFLD<sup>[46-48]</sup>. Recently, it has been demonstrated that 11 $\beta$ -HSD1 expression levels in the liver and in visceral adipose tissue in morbidly obese patients, correlate with dyslipidemia and IR, suggesting that this enzyme might have a pathogenic role in obesity and the related metabolic disorders<sup>[49]</sup>. The role of 11 $\beta$ -HSD1 in NAFLD has been largely studied in humans, with conflicting results<sup>[46,47]</sup>. In any case, in two studies assessing sequential changes of enzyme expression in obese mice developing hepatic steatosis, the Authors found an overexpression of 11 $\beta$ -HSD1 in visceral adipose tissue and hepatic tissue

with the occurrence of portal hypercortisolism<sup>[50,51]</sup>. However, further research is needed to precisely define the role of 11 $\beta$ -HSD1 in the origin and development of NAFLD.

In addition, the effects of treatment with specific 11 $\beta$ -HSD1 inhibitors<sup>[52]</sup> in NAFLD deserve more thorough exploration, as these agents have the potential to improve insulin sensitivity<sup>[53]</sup> and may ultimately add to the available treatment options. Indeed, the rationale behind this type of intervention is challenged by the observation that in NASH, the more severe form of NAFLD, the increased 11 $\beta$ -HSD1 activity and consequent cortisol regeneration is supposed to limit hepatic inflammation<sup>[54]</sup>. This point will be further dealt with later on.

In general, primary pre-adipocyte cultures isolated from human adipose tissue represent heterogeneous cell populations, some of which can be part of the immune system<sup>[55]</sup>. GCs affects the genes associated with immune responses, such as interleukin-6 (IL-6), TNFAIP6<sup>[56]</sup> and CD163. The simultaneous GC-induced downregulation of the TNFAIP6 and IL-6 in human preadipocytes might reflect the interaction between these two genes in adipose tissue inflammation. Glutathione peroxidase 3 precursor (GPX3), the plasma GPX3, catalyses the reduction of hydrogen peroxide, organic hydroperoxide and lipid peroxides, thus protecting cells against oxidative damage. GPX3 was reported to be present in human adipose tissue<sup>[57]</sup>, and GPX3 was identified as being one of the most highly expressed genes in the pre-adipocyte compartment of human adipose tissue as well as a novel GC-target gene.

Recently, adipose tissue has been defined as a major site of production of serum amyloid A (SSA)<sup>[58]</sup>, a well-known risk factor for CAD<sup>[59]</sup>. It has been shown that *ASSA* is a GC-target gene in omental preadipocytes. Together, these findings contribute to the role of omental tissue as a potential link between an inflammatory response and CAD.

## 11 $\beta$ -HSD1 EXPRESSION AND ACTIVITY IN THE LIVER

Conflicting observations have been made regarding this issue. Most studies suggest that 11 $\beta$ -HSD1 expression and activity in the liver is down-regulated in obesity<sup>[54,60]</sup>.

This down-regulation, however, appears to be defective in insulin resistant individuals<sup>[61]</sup>. The failure to down-regulate hepatic 11 $\beta$ -HSD1 could further contribute to IR and, on the basis of the fact that GCs stimulate lipid production, also exacerbate dyslipidemia.

These relationships are complicated by the expression of additional glucocorticoid metabolizing enzymes in the liver, most importantly the A-ring reductases (5 $\alpha$ - and 5 $\beta$ - reductase)<sup>[15,54]</sup>. The expression of these enzymes also appears to be associated with IR and, in a similar manner, to 11 $\beta$ -HSD1, showing a pattern of down-regulation with increased adiposity and insulin resistance. A possible mediator of the hepatic changes seen in the metabolic syndrome could be the increased production of cortisol

from visceral fat in obesity. This increased cortisol would subsequently drain to the liver through portal circulation. However, recent studies examining cortisol and cortisone levels in peripheral, portal, and hepatic vein blood samples indicated that cortisol production from visceral adipose tissue, and thus the amount of exposure of the liver, does not significantly change with increasing obesity<sup>[62-64]</sup>, confirming previous data<sup>[41]</sup>.

## ROLE OF INTERLEUKIN-6

IL-6 is expressed in and released from both the subcutaneous and omental adipose tissues<sup>[65]</sup> with a two- to three-fold higher *in vitro* release of IL-6 from omental compared to subcutaneous adipocytes *in vitro*. The *in vivo* release of IL-6 from fat contributes as much as 35% to the basal circulating levels and may at least in part explain the positive correlation between serum levels of IL-6 and obesity. In rodents, obesity is associated with macrophage accumulation in adipose tissue, and these macrophages release inflammatory mediators and molecules promoting inflammation. Inflammatory mechanisms play a key role in the pathogenesis of type 2 diabetes. This low-grade chronic inflammation has been proposed to be mediated by the IL-6 family of cytokines, tumor necrosis factor- $\alpha$ , interleukin-1 beta, IL-18, and certain other chemokines. In addition to its immunoregulatory actions IL-6 has been proposed to affect glucose homeostasis and metabolism directly and indirectly by its action on skeletal muscle cells, adipocytes, hepatocytes, pancreatic beta-cells and neuroendocrine cells<sup>[66]</sup>. It has been suggested that IL-6 might play a role in the pathogenesis of Cushing's disease<sup>[67]</sup>. Although an increased production of IL-6 has been reported in patients with either active or remitted Cushing's syndrome, elevated GCs levels in these patients may prevent excessive action of IL-6 due to the persistent accumulation of central fat<sup>[68]</sup>. In fact, it has been hypothesized that IL-6 deficiency exacerbates hepatic insulin resistance and inflammation, a process that appears to be related to defects in mitochondrial metabolism<sup>[69]</sup>. On the other hand, higher levels of circulating IL-6 were found in patients with the more severe form of NAFLD<sup>[70]</sup>.

IR is strictly linked to anti-apoptotic serum Bcl-2 values<sup>[71]</sup>, which were higher in simple fatty liver than in NASH patients, suggesting a protective role of the anti-apoptotic process in liver and perhaps in other areas<sup>[72]</sup>. Apoptotic cell death is caspase-dependent and is associated with mitochondrial membrane depolarization and cytochrome c release<sup>[73]</sup>. Adhering to the hypothesis that the exposure of hepatocytes to free fatty acids, resulting in increased reactive oxygen species production and mitochondrial damage, is balanced by the presence of anti-oxidant substances, circulating levels of gamma-glutamyl transferase, cytochrome c, triglycerides and unconjugated bilirubin were explored in patients suffering from non-alcoholic fatty liver disease with different severity<sup>[73]</sup>. The resulting findings likely reflect a balance between oxidative stress and anti-oxidant response rather than a lack

of reliability of cytochrome c as a reliable biomarker of mitochondrial damage<sup>[73]</sup>.

The inflammatory mediators that are biosynthesized in the liver and increased in NAFLD patients include C-reactive protein (CRP)<sup>[74]</sup>, IL-6<sup>[70]</sup>, fibrinogen and plasminogen activator inhibitor-1 (PAI-1)<sup>[75]</sup>. Consequently, fat in the liver represents a site beyond adipose tissue that independently contributes to the inflammatory process. In support of a certain sequence of cellular and molecular events that mediates hepatic IR in NAFLD, recent data lend credence to the fact that hepatic steatosis activates I $\kappa$ B kinase (IKK)- $\beta$  and nuclear factor (NF)- $\kappa$ B<sup>[76]</sup>. Among the inducible transcription factors that control inflammatory gene expression, NF- $\kappa$ B plays a central and an evolutionarily conserved role in coordinating the expression of various soluble pro-inflammatory mediators (cytokines and chemokines) and leukocyte adhesion molecules. In non stimulated cells, NF- $\kappa$ B is sequestered in cytosol by the inhibitor of NF- $\kappa$ B (I $\kappa$ B), which masks the nuclear localization signal present along the NF- $\kappa$ B protein sequence.

Treatment of cells with pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1, or with bacterial products such as lipopolysaccharide, leads to the activation of a specific-IKK complex that phosphorylates I $\kappa$ B and thereby tags it for ubiquitination and degradation by the proteasome<sup>[77]</sup>. The degradation of I $\kappa$ B thus allows NF- $\kappa$ B to translocate into the nucleus where it can act as a transcription factor that upregulates IL-6 production and secretion. IL-6 works locally through paracrine and/or endocrine mechanisms to activate IL-6 signaling in the liver. IL-6 is known to induce IR in hepatocytes<sup>[78]</sup>. Hepatic production of IL-6 also provides a further pathogenic link to extrahepatic organs such as muscle. NF- $\kappa$ B target genes are not upregulated in transgenic mouse muscle, unlike IL-6 target genes and the suppressor of cytokine signaling and signal transducer and activator of transcription proteins. These genes are reversed during IL-6 neutralization, which is consistent with the pathogenic involvement of IL-6. Activation of NF- $\kappa$ B leads to a severe syndrome of muscle wasting, without IR<sup>[79]</sup>.

Fat mass in overweight/obese subjects has a primary role in determining low-grade chronic inflammation and, in turn, IR and ectopic lipid storage within the liver<sup>[80]</sup>. Obesity, aging, and fat mass all influence the growth hormone/insulin-like growth factor (IGF)-I axis, and chronic inflammation might reduce IGF-I signaling. Altered IGF-I axis is frequently observed in patients with hepatic steatosis<sup>[80]</sup>. In our study population, lower IGF-I status is associated with higher fat mass, spleen longitudinal diameter, CRP and more severe hepatic steatosis<sup>[80]</sup>.

## 11 $\beta$ -HSD1 IN INFLAMMATION

At least some of the immunomodulatory effects of GCs in the inflammatory response are dependent on 11 $\beta$ -HSD1 activity. For example, 11 $\beta$ -HSD1-deficient mice suffering from experimental arthritis exhibit a de-

layed resolution of the inflammatory response, probably due, in part, to attenuated macrophage phagocytosis of leukocyte apoptotic bodies<sup>[81,82]</sup>. As GCs regulate both the suppression of the early phase and the promotion of the late phase of the inflammatory response, it is conceivable that in generally deregulated - *i.e.*, both decreased and increased - GC levels could contribute to chronic inflammatory disease. Even if not characteristically for all chronic inflammatory conditions, some of them have been associated with increased 11 $\beta$ -HSD1 expression, in particular inflammatory diseases of the digestive tract, such as inflammatory bowel disease and colitis<sup>[82-85]</sup>, as well as atherosclerosis<sup>[44,86]</sup>. These observations are in line with several reports evidencing the induction of 11 $\beta$ -HSD1 expression by pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  in various cell types and lines including fibroblasts, adipocytes, osteoblasts and smooth muscle cells<sup>[5-7,87-91]</sup>.

## PARADOX OF IL-6 IN HUMANS AND ANIMALS

If global deletion of IL-6 not only develops obesity, but also hepatosteatosis and liver inflammation in animal model<sup>[69]</sup>, what is that determines the low prevalence of hepatic steatosis in patients suffering from Cushing's disease<sup>[22]</sup>?

Ahmed *et al.*<sup>[54]</sup> defined hepatic GCs metabolism in progressive NAFLD, which can be summarized into two distinct phases of altered regulation of hepatic cortisol metabolism: (1) increased hepatic cortisol clearance in steatosis; and (2) increased hepatic cortisol regeneration in NASH. Failure to regulate in this way may worsen the phenotype of liver disease driving hepatic steatosis or unchecked progressive hepatic inflammation<sup>[54]</sup>.

Considering the broadly accepted presence of inflammatory elements in the etiology of obesity, 11 $\beta$ -HSD1 probably plays an important causative role in the development of the metabolic syndrome, positioning itself at the interface of inflammation, hepatic steatosis and Cushing's syndrome.

From another perspective, under conditions that avoided changes in food intake, the efficacy of 11 $\beta$ -HSD1 inhibition to up-regulate hepatic fat oxidation gene expression - which reduces the glucocorticoid effects in liver and fat - functionally translates into enhanced hepatic lipid oxidation *in vivo*<sup>[92]</sup>.

## ANSWERED QUESTIONS AND CONCLUDING REMARKS

Considering the 50% five-year survival rate of patients with endogenous Cushing's syndrome (without any treatment)<sup>[93]</sup> and the fact that all patients enrolled into the study by Rockall *et al.*<sup>[22]</sup> were all newly diagnosed patients, should we discuss the question of time exposure as a possible explanation for the relatively low prevalence of

hepatic steatosis in patients with endogenous Cushing's syndrome? Should the relative hypercortisolemia (the increase in free cortisol or circadian disturbance) in patients with metabolic syndrome not be completely disregarded? At least two diagnostic studies aiming to evaluate patients with Cushing's syndrome among patients with obesity have found that late-night salivary cortisol (free cortisol) is increased in patients with obesity. Baid *et al.*<sup>[94]</sup> reported that late night salivary cortisol was frequently above the laboratory-provided reference range in obese and overweight subjects ( $n = 369$ ). In another paper late-night salivary cortisol was statistically significantly increased in patients with obesity and some features of Cushing's syndrome, in whom endogenous Cushing's syndrome was excluded, *vs* healthy, normal BMI control subjects<sup>[95]</sup>. Since IL-6 also plays an anti-inflammatory activity, it seems reasonable to assume that favorable aspects exist, such as inactivating proinflammatory mediators that induce the production of cortisol during exercise, and thus influence insulin sensitivity, with enhanced insulin action at muscle level, immediately at early recovery<sup>[96]</sup>. Could this anti-inflammatory effect of high circulating GCs affect hepatic steatosis in Cushing's disease?

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## Surveillance for hepatocellular carcinoma in chronic liver disease: Evidence and controversies

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### Abstract

Primary liver cancer is the sixth most common cancer in the world and the third cause of cancer-related death. Hepatocellular carcinoma (HCC) represents more than 90% of primary liver cancers and generally occurs in patients with underlying chronic liver disease such as viral hepatitis, hemochromatosis, primary biliary cirrhosis and non-alcoholic steatohepatitis. Especially cirrhotic patients are at risk of HCC and regular surveillance could enable early detection and therapy, with potentially improved outcome. We here summarize existing evidence for surveillance including ultrasound, other radiological modalities and various serum biomarkers, and current international guideline recommendations for surveillance. Ultrasound and  $\alpha$ -fetoprotein (alone or in combination) are most frequently used for surveillance, but their sensitivities and specificities are still far from perfect, and evidence for surveillance remains weak and controversial. Various other potential surveillance tools have been tested, including serum markers as des-car-

boxyprothrombin, lectin-bound  $\alpha$ -fetoprotein, and (most recently) circulating TIE2-expressing monocytes, and radiological investigations such as computed tomography-scan or magnetic resonance imaging-scan. Although early results appear promising, these tools have generally been tested in diagnostic rather than surveillance setting, and in most cases, no detailed information is available on their cost-effectiveness. For the near future, it remains important to define those patients with highest risk of HCC and most benefit from surveillance, and to restrict surveillance to these categories.

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**Key words:** Hepatocellular carcinoma; Surveillance; Chronic liver disease

**Core tip:** Hepatocellular carcinoma is a frequent phenomenon in cirrhotic patients. Survival is generally poor, and curative options only exist if the tumor is detected in an early stage (Barcelona Clinic Liver Cancer stage 0 or A). This review summarizes existing evidence for surveillance including ultrasound, other radiological modalities and various serum biomarkers, and current guideline recommendations for surveillance. Selection of the appropriate high risk populations remains an important tool for cost-effective surveillance.

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### INTRODUCTION

Primary liver cancer is the sixth most common cancer

in the world and the third cause of cancer-related death. Hepatocellular carcinoma (HCC) represents more than 90% of primary liver cancers and generally occurs in patients with underlying chronic liver disease. Incidence rates are highest in East Asia and Sub-Saharan Africa, where approximately 85% of all cases occur. The high HCC risk in these regions can be explained at least in part by the high prevalence of underlying risk factors, especially chronic hepatitis B virus (HBV) infection and aflatoxin B1 in the diet<sup>[1,2]</sup>.

Nevertheless, also in countries with relatively low incidence rates such as the United States, incidences have doubled over the last two decades, due to growing impact of chronic liver disease from hepatitis C virus (HCV) and non-alcoholic steatohepatitis (NASH)<sup>[3,4]</sup>. In Europe, there is a mixed pattern, with increasing or decreasing incidence rates in various countries. These geographical differences are thought to be the consequence of changing patterns of underlying risk factors for HCC, such as viral hepatitis, alcohol abuse and NASH. Although NASH conveys relatively modest risks of HCC, the burden of metabolic syndrome and insulin resistance is expected to have significant further impact on HCC incidence in the Western world in the near future. Also, coexistent metabolic syndrome and obesity further increase HCC risk in patients with other underlying liver diseases<sup>[5-7]</sup>. Smoking is another cofactor leading to increased HCC risk<sup>[7]</sup>. Interestingly, the use of cholesterol synthesis inhibitors and metformin and coffee consumption are associated with decreased HCC risk<sup>[8-11]</sup>.

New local treatment options such as radiofrequency ablation (RFA) or transarterial chemo- and radioembolisation (TACE and TARE) have been introduced in recent years. Also, the multikinase inhibitor sorafenib leads to improved survival in patients with advanced stage disease<sup>[12]</sup>. Nevertheless, most patients present at relatively late stage, without options for curative treatment. Survival remains poor under these circumstances [median survival: Barcelona Clinic Liver Cancer (BCLC) stage B, 15 mo; BCLC stage C, 6 mo; BCLC stage D, < 3 mo]<sup>[13]</sup>. Therefore, new strategies are urgently needed to decrease the burden of HCC. First, primary prevention of HCC can be achieved by hepatitis B vaccination, especially in endemic countries<sup>[14]</sup>. Second, antiviral therapies appear to be associated with decreased HCC risks in patients with chronic viral hepatitis. Interestingly, according to recent meta-analyses and systematic reviews, interferon-based antiviral treatment for hepatitis B is associated with only modest decrease of HCC risk (RR = 0.66, 95%CI: 0.48-0.89). Treatment with the nucleoside analog lamivudine is associated with a more substantial risk reduction (RR = 0.22, 95%CI: 0.10-0.50)<sup>[15-17]</sup>. It is reasonable to expect even better results with the currently available potent nucleos(t)ide analogs tenofovir and entecavir<sup>[18]</sup>.

Third, and focus of the current review, surveillance of patients at increased risk because of underlying chronic liver disease might detect HCC at earlier stages, and

could lead to better outcomes with decreased mortality (5-year survival with curative treatment in BCLC stage 0 or A: 40%-70%)<sup>[13]</sup>. Intuitively, this approach is attractive. Indeed, several international guidelines currently advise surveillance programs, defined as the repeated and systematic administration of a screening test, including registration and patient recall where applicable<sup>[19-21]</sup>. Nevertheless, surveillance remains controversial because of limited evidence for its efficiency and potential risk of side effects (for example complication from diagnostic biopsy of liver mass detected during surveillance which subsequently proves to be benign)<sup>[22,23]</sup>. This review offers an overview of current knowledge on HCC surveillance and gives a perspective on current international guidelines.

## BIOMARKERS FOR HCC SURVEILLANCE

When introducing a surveillance program, applying the optimal screening modality is essential. Detection of HCC at intermediate or advanced stage [according to Barcelona Clinic Liver Cancer Staging (BCLC) system] by surveillance could have some impact, by allowing TACE or sorafenib therapy. Nevertheless, surveillance should preferably detect (very) early stage HCC (single lesion  $\leq$  5 cm or  $\leq$  3 lesions each  $\leq$  3 cm without vascular involvement or metastasis), allowing potentially curative therapy. Serological biomarkers can be used at relatively low costs, without burden for the patient. Several biomarkers have been investigated for HCC detection, generally in diagnostic setting rather than in surveillance studies.

$\alpha$ -fetoprotein (AFP) is the most frequently used biomarker for HCC worldwide. AFP is a glycoprotein expressed by fetal hepatocytes or poorly differentiated HCC cells. Nevertheless, not all HCC cells secrete AFP into the circulation. Also, serum AFP levels may be elevated in patients with chronic liver disease in the absence of HCC (related to height of transaminases) and in patients with other malignancies<sup>[24]</sup>. Gupta *et al.*<sup>[25]</sup> published a systematic review with five included studies on diagnostic value of AFP for detecting HCC (all stages combined) in cirrhotic and non-cirrhotic HCV patients. They concluded that even if one assumes best-case estimates of sensitivity and specificity, the case for surveillance by AFP remains weak. By using the usual cut-off point of 20 ng/mL, sensitivities and specificities for detecting all stages of HCC were 41%-65% and 80%-94%, respectively. Positive and negative likelihood ratios ranged from 3.1 to 6.8 and from 0.4 to 0.6, respectively. In a recent large case-control study with 419 included HCC patients and 417 cirrhotic controls (with various underlying etiologies of liver disease), the performance of AFP in early stage HCC was compared with other biomarkers, such as des-carboxyprothrombin (DCP) and lectin-bound AFP, with more encouraging results<sup>[26]</sup>. In this study, diagnosis of HCC was based on current criteria for diagnosis, with appropriate blinding and 6-mo post-enrollment follow-

up for controls to eliminate false negatives. Sensitivity and specificity of AFP for detecting early stage HCC were 53% and 90%, respectively, using the currently recommended clinical cut-off point of 20 ng/mL. When the point in the receiver operating characteristics (ROC) curve with optimal sensitivity and specificity was used as cut off (10.9 ng/mL), sensitivity was 66% and specificity 82%. This finding would suggest that the usual cut off of 20 ng/mL is too high for optimal performance of AFP surveillance. In a previous small case-control study with 170 HCC patients (68 early stage HCC) and 170 matched cirrhotic controls, the cut off of 20 ng/mL appeared to exhibit optimal sensitivities and specificities<sup>[27]</sup>. Nevertheless, at this cut off, sensitivities and/or specificities were often insufficient in other studies<sup>[28,29]</sup>.

DCP is an abnormal protein generated as a result of an acquired defect in the posttranslational carboxylation of the prothrombin precursor in malignant liver cells<sup>[30]</sup>. Several studies investigated the performance of DCP as biomarker for early HCC, with inconclusive results<sup>[26,31-34]</sup>. For example, in a case-control study with 39 HCC patients and 77 matched controls, DCP had a greater accuracy than AFP<sup>[34]</sup>. DCP testing alone had a sensitivity and specificity of 74% and 86%, respectively, using a cutoff value of 40 mAU/mL. Another study reported much lower sensitivities for DCP with the best performance in chronic viral hepatitis<sup>[26]</sup>.

Another potential biomarker for HCC is lectin-bound AFP: One of the three glycoforms of AFP, based on its reactivity in lectin affinity electrophoresis<sup>[35]</sup>. In a multicenter prospective study, the diagnostic accuracy of lectin-bound AFP *vs* AFP was compared in patients with HCV-related cirrhosis<sup>[36]</sup>. The prevalence of HCC at baseline and during the two years of follow-up was significantly higher in patients with elevated lectin-bound AFP than in those with elevated AFP. The relatively high prognostic value of lectin-bound AFP was even higher in patients with concomitantly elevated AFP levels. This suggests that lectin-bound AFP has some clinical utility as secondary test in HCV patients with mildly elevated AFP levels, by identifying a subgroup with a relatively high likelihood of HCC. Nevertheless, this study has important limitations, such as a relatively short follow-up and potential selection bias. Also, other investigators report less encouraging results. For example, in the previously mentioned case-control study of Marrero *et al.*<sup>[26]</sup>, sensitivity of lectin-bound AFP for detecting early HCC was only 37%. Several studies investigated the diagnostic performance of a combination of serum biomarkers. Nevertheless, when combining AFP and DCP, there appeared to be only little or no improvement in sensitivity rates for detecting early stage HCC<sup>[26,33,34]</sup>.

Recently, new serum biomarkers for HCC have been suggested. Tyrosine kinase with Ig and EGF homology domains 2 (TIE2) is a receptor of angiopoietins. TIE2-expressing monocytes (TEMs) were recently reported to be enriched in HCC and other tumors where angiogenesis is known to be important for tumor progression. In

a recent publication on 168 HCV infected patients (89 with HCC), frequency of circulating TEMs in peripheral blood was significantly higher in case of HCC, and independent of tumor stage<sup>[37]</sup>. TEMs were also increased in a separate group of non-HCV HCC patients. Performance of TEMs in discriminating HCC from chronic hepatitis or cirrhosis was superior to AFP or DCP (sensitivities 86% and 71% respectively; specificities 81% and 90% respectively). Nevertheless, another study found increased circulating and intrahepatic TEMs in HCV patients without HCC<sup>[38]</sup>. Although these findings relate to a relatively small cohort of HCV-infected patients, they raise concern that mobilization and expansion of TEMs may not be strictly HCC-driven, but more generally associated with chronic liver infection<sup>[39]</sup>. Several other studies investigated the performance of Glypican-3 (GPC3). GPC3 is a surface protein expressed in high percentages of HCCs, whereas it is not detectable in hepatocytes from normal subjects or patients with benign liver disease<sup>[40-42]</sup>. Another potential marker is Golgi protein 73 (GP73): An amino acid that normally remains in the Golgi complex. Marrero *et al.*<sup>[43]</sup> reported that levels of GP73 are increased in serum of patients with HCC. In this study, sensitivity of GP73 for detecting early HCC was 62%. Also, interleukin-6 (IL-6) has been studied as potential marker for HCC. IL-6 is a cytokine involved in cell growth and differentiation. Serum IL-6 concentrations appeared to be increased in HCC patients (all stages combined) compared to controls<sup>[44,45]</sup>. Sensitivities of IL-6 for discrimination between HCC patients (all stages combined) and controls ranged from 46% to 73% and specificities from 87% to 95%. Also, levels of squamous cell carcinoma antigen (SCCA: A component of serine protease inhibitors) appeared to be significantly higher in HCC patients than in controls<sup>[46,47]</sup>. It remains to be seen, whether these new serum biomarkers will provide satisfactory results in the setting of surveillance in clinical practice.

## ULTRASONOGRAPHY FOR HCC SURVEILLANCE

Currently, ultrasonography (US) is the most widely used method for HCC surveillance. US is not invasive, but time-consuming, relatively expensive and operator-dependent. Also, this investigation is often not suitable in case of overweight. According to a recent meta-analysis with 13 included studies by Singal *et al.*<sup>[48]</sup>, pooled sensitivities and specificities for detecting HCC at any stage were both 94%. However, US was less effective for detecting early stage -potentially curable- HCC, with a pooled sensitivity of 63% (95%CI: 49%-76%).

Another systematic review concluded that US is insufficiently sensitive for HCC surveillance<sup>[49]</sup>. Sensitivities for detecting HCC (all stages combined) of the 14 included studies ranged from 30% to 100% and specificities from 73% to 100%. Possible explanations for the large variability between studies are differences in opera-

tor skills and experience, in tested populations and/or in tumor size.

The potential benefit of combining US with AFP for detection of early stage HCC was also explored in the previously mentioned meta-analysis by Singal *et al*<sup>[48]</sup>. The pooled sensitivities increased from 63% to 69% (95%CI: 53%-81%), but this was not statistically significant ( $P = 0.65$ ). This result suggests that adding AFP to ultrasound is not very useful for HCC surveillance.

The optimal interval for ultrasonographic surveillance is not known. It should be based on rate of tumor growth up to the limit of its detectability: Available evidence on tumor growth suggests that the interval from undetectable to a two cm diameter lesion ranges from 4-12 mo<sup>[50]</sup>. According to the meta-analysis by Singal *et al*<sup>[48]</sup> mentioned above, US sensitivities for detecting early-HCC may be improved by US at 6-mo intervals compared to surveillance intervals between 6-12 mo (pooled sensitivities: 70% *vs* 50%,  $P = 0.001$ ). However, confidence intervals of pooled sensitivities were overlapping (95%CI: 55.6-84.6 *vs* 40.0-59.2).

Two recent retrospective cohort studies from Italy and South Korea report that HCC is detected at earlier stage, with curative therapy more often applied and better survival in case of surveillance interval  $\leq 6$  mo compared to longer surveillance intervals, even after correction for lead time bias<sup>[51,52]</sup>.

A recent randomized control trial investigated whether a 3-mo interval of US surveillance was more effective than a 6-mo interval<sup>[53]</sup>. More focal lesions  $< 10$  mm were found in the group with 3-mo surveillance (5-year cumulative incidence: 41% *vs* 28%,  $P = 0.002$ ). Nevertheless, 44% of all focal liver lesions detected during surveillance remained indeterminate. No differences in detection rates of small HCCs eligible for curative treatment were observed between the two randomized groups. Inadequate compliance occurred in approximately 10% of both groups. Also, overall 5-year survival rates were similar in both groups (85% *vs* 86%,  $P = 0.38$ ).

Finally, a recent cluster-randomized trial from Taiwan compared 4- and 12-mo intervals in chronic HBV or HCV patients with thrombopenia. Although tumors were smaller in the group with 4-mo intervals, and curative treatment modalities more often applied, 4-year overall survival did not differ<sup>[54]</sup>.

## OTHER IMAGING TECHNIQUES FOR HCC SURVEILLANCE

Computed tomography (CT) and magnetic resonance (MR) imaging are potential tools for HCC surveillance. Until now, these imaging modalities are mainly used for further evaluation in case of abnormal findings with ultrasonographic surveillance and to determine extent of disease. Test characteristics of CT and MR imaging reported below are therefore all based on diagnostic studies rather than in a setting of surveillance. According to a systematic review of Colli *et al*<sup>[49]</sup> (studies in the period:

1996-2004 included), spiral CT imaging appeared to offer comparable sensitivities and specificities as US for detecting HCC (all stages) in patients with chronic liver disease. The pooled sensitivities for US and spiral CT imaging were 60% *vs* 68% and the pooled specificities 97% *vs* 93%. In the same systematic review, the reported pooled sensitivities (81%) and specificities (85%) of magnetic resonance imaging (MRI)-scan were, respectively, higher and lower than those obtained with US or CT imaging.

However, diagnostic accuracies of CT-scan and MRI-scan have increased in the past decade due to improvement of techniques<sup>[55-58]</sup>. For example, according to a recent retrospective study, the overall sensitivities of triple-phase multidetector CT (MDCT) imaging for detecting HCC (all stages combined) ranged between various observers from 78% to 81%<sup>[57]</sup>. Sensitivity improved with increasing HCC diameter. Also, in another recent study with prospective design, the diagnostic performances of US, MDCT imaging and contrast-enhanced MRI-scan were compared in a population of cirrhotic candidates for liver transplantation<sup>[58]</sup>. Dynamic MRI-scan with inclusion of the hepatobiliary phase had the highest accuracy with sensitivities for detecting early stage HCC, ranging from 59% to 85%. In contrast to CT imaging, MRI-scan is not associated with radiation exposure. However, MRI-scan is costly and there are no data from clinical practice to support the use of MRI-scan for surveillance.

## SURVEILLANCE EFFICIENCY

The aim of surveillance is to decrease HCC-related mortality. Unfortunately, high-level evidence is limited in this respect. A recent Cochrane review<sup>[59]</sup> identified only one study with data on mortality: Zhang *et al*<sup>[60]</sup> performed a large cluster randomized controlled trial in which a policy of surveillance *vs* no surveillance was compared in 18816 patients with current or prior HBV infection or a history of chronic hepatitis. There proved to be a survival benefit for the strategy of a 6-monthly surveillance with combined AFP and US compared to the no surveillance strategy. Despite poor adherence to surveillance (58%), HCC-related mortality rates were significantly lower in the surveillance group than in the disease control group (83.2/100000 and 131.5/100000, respectively, OR = 0.63). Since this study was performed in chronic HBV patients in China, it is not clear whether its results can be extrapolated to the Western World. Also, according to the Cochrane review, there are some discrepancies between this publication and earlier publications about the same trial<sup>[61-63]</sup>. For example, numbers of disease controls and total numbers of participants dropped from 9711 and 19144, respectively, in the initial preliminary publication in 1997<sup>[62]</sup> to 9443 and 18816, respectively, in two later publications in 1999 and 2004, after completion of the trial<sup>[60,61]</sup>. Also, confidence intervals were computed as if cohorts had been randomly assigned for each individual patient, without taking the cluster randomization into account and statistical significance

was claimed with a 95%CI that was only borderline significant (95%CI: 0.41-0.98)<sup>[22]</sup>. In another large randomized controlled trial from China, the effectiveness of surveillance by 6-monthly AFP measurement in 5581 HBV carriers was investigated<sup>[29]</sup>. HCC-related mortality rates were not significantly different in the surveillance group compared to the control group (1138/100000 and 1114/100000, respectively,  $P = 0.86$ ).

At lower evidence level, several cohort studies suggest that survival is improved with HCC surveillance<sup>[64,65]</sup>. In a population-based study of Alaska natives, 1487 HBV carriers had surveillance with AFP at 6-mo intervals<sup>[64]</sup>. In case of elevated AFP, US was performed. The long-term survival rate for patients whose HCCs were detected by the surveillance program was compared with a historical disease control group of patients with HCC: Survival rates were significantly higher for patients with HCC detected by surveillance (5-year survival rates: 42% and 0%, respectively,  $P = 0.008$ ; 10-year survival rates: 30% and 0%, respectively,  $P = 0.07$ ). Similar results were found in the study of Wong *et al*<sup>[65]</sup>. In this study, 56 HCC patients were retrospectively divided in three groups according to their initial presentation: Symptomatic patients presenting with abdominal pain, mass, bleeding, or weight loss; asymptomatic patients who had ultrasound for abnormal liver enzyme levels or other (unrelated) indications; and asymptomatic patients with underlying chronic liver diseases in a surveillance program. Patients in the surveillance group survived significantly longer than those in the symptomatic group (median survival of 1300 d *vs* 234 d,  $P = 0.009$ ). Median survival of the asymptomatic group without surveillance did not differ significantly from the other groups. However, lead time bias due to disease detection in an early stage could have affected the results of the two previously mentioned studies. Also, length time bias could have biased results by preferential detection of slowly growing lesions that are more likely to remain asymptomatic until late in disease course.

Several studies indicate that surveillance programs could lead to more frequent detection of HCC at early stages, when curative treatment is still possible<sup>[60,61,66,67]</sup>. In the previously mentioned large randomized controlled trial by Yang *et al*<sup>[61]</sup>, more resectable HCC cases were detected in the surveillance group than in the disease control group during five years follow-up (OR = 7.14; 95%CI: 3.53-14.43). In the later publication about the same trial, Zhang *et al*<sup>[60]</sup> reported similar results. Again, detection rates of small HCC (defined as a tumor diameter < 5 cm) were higher in the surveillance group than in the disease control group (45% *vs* 0%,  $P < 0.01$ ).

In the systematic review by Gebo *et al*<sup>[66]</sup> covering publications in the period: 1996-2002, one prospective cohort study was identified that investigated HCC surveillance in HCV patients. This study by Solmi *et al*<sup>[67]</sup>, compared 360 patients in the surveillance group (combined US and AFP measurements at 6-mo intervals) with a disease control group of 2170 patients who received usual care in other hepatology clinics. During

a mean follow-up of 56 mo, the incidence of HCC in the surveillance group was 6.7% and 5.5% in the disease control group. Of note, in the surveillance group, 75% of the HCC's was unifocal and  $\leq 3$  cm in diameter compared to only 16% in the disease control group ( $P = 0.000$ ).

Success of a surveillance program depends in general not only on the surveillance modality or target population. Recall strategy and adherence to follow-up are also important factors. In a large retrospective cohort study in the United States, 1873 HCC patients with a prior diagnosis of cirrhosis were identified in the period: 1994-2002<sup>[68]</sup>. In the three years before HCC diagnosis, 17% received regular surveillance, 38% received inconsistent surveillance and 45% no surveillance. In the regular surveillance group, 52% received both US and AFP, 46% AFP only and 2% US only. In a subset of 541 patients in whom cirrhosis was diagnosed at least three years prior to HCC, 29% received regular surveillance and 33% inconsistent surveillance. In another prospective cohort study, 1005 HCV patients were included in a surveillance program with combined US and AFP at 6- to 12-mo intervals<sup>[69]</sup>. During a mean follow-up of 6.1 years, 83 HCC cases were detected: 28% of those were tumors outside Milan criteria. 70% of patients with HCCs outside Milan criteria had experienced consistent surveillance and follow-up, which was not different from the total study group. Only in a minority of patients, absence of surveillance (13%) or follow-up (17%) could explain failure to detect patients at earlier stages. On multivariate analysis, study site was a strong independent predictor of consistent surveillance ( $P < 0.001$ ). After correction for study site, also platelet counts  $> 150 \times 10^6/\text{mL}$  and complete clinic visit adherence were positively associated with consistent surveillance.

## TARGET POPULATIONS FOR SURVEILLANCE

Most HCCs develop in patients with chronic liver diseases. The decision to start surveillance should depend on the chance of developing HCC, *i.e.*, the incidence of HCC in specific populations. Especially high risk patients should be included in surveillance programs. However, selection of these patients remains a subject of debate. Also, it is important to decide, prior to surveillance, whether the clinical condition of the patient would allow any therapy in case of HCC detection.

### Computer analyses in hypothetical cirrhotic patients

Several studies have investigated cost-effectiveness of HCC surveillance in cirrhotic patients based on computer analyses of theoretical models. In general, surveillance is considered cost-effective, if costs are less than \$50000 per life-year or quality-adjusted life-year (QALY) gained. According to Sarasin *et al*<sup>[70]</sup>, surveillance with combined US and AFP measurements at 6-mo intervals in patients with compensated cirrhosis results in an increase of life

expectancy of 3 mo when HCC incidence is 1.5%/year. However, in this case cost-effectiveness ratio of \$55264 per life year gained exceeds the generally accepted threshold of \$50000 per life year gained. When HCC incidence rates are higher, increase of life expectancy will be even more substantial. Cost-effectiveness ratios of systematic surveillance range between \$26000 (HCC incidence: 6%/year) and \$55000 (HCC incidence: 1.5%/year) for each additional life-year gained, in best case scenarios. Another study, using similar analyses in HCV cirrhotics, suggested that HCC surveillance with combined three-phase CT-scans and AFP measurements at 6-mo intervals was more cost-effective than other surveillance strategies<sup>[71]</sup>. This strategy was associated with an incremental cost-utility ratio of \$25232/QALY compared to no surveillance. These results are based on estimated HCC incidences of 1.4%/year in patients with compensated cirrhosis and 4%/year in patients with decompensated cirrhosis.

Lin *et al.*<sup>[72]</sup> suggested that combined US and AFP measurements at 6-mo intervals was the most effective surveillance strategy in HCV patients with compensated cirrhosis compared to other surveillance strategies (estimated annual HCC incidence: 0.02%-0.1%). However, this strategy entailed higher additional cost per QALY or life-year gained compared to a strategy of annual US and AFP measurements (incremental cost-effectiveness ratio: \$106871/QALY) or no surveillance (incremental cost-effectiveness ratio: \$129915/QALY). In this computerized decision model, surveillance with US at 12-mo intervals and AFP measurements at 6-mo intervals offered the greatest gain in life-expectancy, while still maintaining a cost-effectiveness ratio < \$50000/QALY or life-year gained, regardless of HCC incidence.

Another study in a hypothetical mixed-etiology cohort of compensated cirrhotic patients suggested that combined AFP measurement and US imaging on a 6-monthly basis is the most effective surveillance strategy<sup>[73]</sup>. Compared to no surveillance, this strategy is estimated to increase the numbers of HCCs resectable at time of diagnosis more than three-fold with 50% decrease of HCC-related deaths. Significantly more small and medium-sized HCCs (diameter < 2 cm or diameter 2-5 cm, respectively) would be identified by surveillance. However, when costs were taken into account, it was doubtful whether US should be routinely offered to those with serum AFP levels ≤ 20 ng/mL (> \$45000/QALY). Also, cost-effectiveness varied considerably depending on type of underlying chronic liver disease. In this computerized decision model, annual HCC incidence ranged from 1.2% to 4.1%.

### HCC incidence in cirrhotic patients

Annual incidence rates of HCC in specific populations with cirrhosis have been investigated extensively. Cirrhotic HBV patients have an increased risk for developing HCC. Studies that included only East Asian cirrhotic HBV patients showed HCC incidence rates around 3.2 per 100 person-years and 5-year cumulative HCC inci-

dence of 15%<sup>[74,75]</sup>. Studies in European HBV cirrhotics reported lower incidence rates: 2.2 per 100 persons-years and 5-year cumulative HCC incidence of 9%<sup>[76-79]</sup>.

Several studies have investigated the risk of developing HCC in HCV patients<sup>[76,80-83]</sup>. In a large prospective study of 12008 Taiwanese males, anti-HCV positive subjects had a 20-fold increased risk of developing HCC in comparison with anti-HCV negative subjects<sup>[81]</sup>. In this study, the presence or absence of cirrhosis was not evaluated. Other studies reported that especially cirrhotic HCV patients are at increased risk for developing HCC<sup>[76,80,83]</sup>. In a retrospective cohort study, the annual HCC incidence was 1.4% in European cirrhotic HCV patients<sup>[76]</sup>. Also, Degos *et al.*<sup>[83]</sup> reported HCC incidence rates of 13.4% after five years follow-up in a cohort of French patients with compensated HCV-related cirrhosis. In another study from Germany, 17 of 838 HCV patients (2%) developed HCC during a mean follow-up of 50 mo<sup>[80]</sup>. All HCCs occurred in cirrhotic livers. Cirrhotic patients had a 20-fold higher risk of developing HCC than HCV patients without cirrhosis at study entry (RR = 20.2, 95%CI: 2.4-170.9). Also, Lok *et al.*<sup>[82]</sup> reported that HCC incidence was higher in HCV patients with cirrhosis than in those with bridging fibrosis (annual HCC incidence rates: 1.45% *vs* 0.8%, *P*<sub>trend</sub> = 0.08). This study was performed in the United States.

In patients with cirrhosis due to causes other than viral hepatitis, HCC incidence rates are generally not precisely known. Nevertheless, alcoholic liver disease is a clear risk factor for HCC. In a meta-analysis of Bagnardi *et al.*<sup>[84]</sup>, the consumption of alcohol was associated with an increased risk for developing liver cancer (RR = 1.86, 95%CI: 1.53-2.27 for alcohol consumption of 100 g/d compared to no alcohol). The presence or absence of cirrhosis was not evaluated in this meta-analysis. In a Danish nationwide cohort study, 169 (2%) of 8482 patients with alcoholic cirrhosis developed HCC during a median follow-up of 4.1 years<sup>[85]</sup>. Five-years cumulative HCC risk was 1.0% (95%CI: 0.8-1.3). Kuper *et al.*<sup>[86]</sup> reported a relative risk of HCC of 2.4 for alcoholism alone and 22.4 for alcoholic cirrhosis compared to the general population. Several other studies also reported increased HCC risk in cirrhotic patients with alcohol as primary cause<sup>[87-91]</sup>. In a case-control study, heavy alcohol consumption contributed to a significant part of the 115 included HCC cases (32%), independent of other known risk factors<sup>[92]</sup>.

Patients with cirrhosis due to genetic hemochromatosis also are at increased risk of developing HCC. According to a meta-analysis, patients with genetic hemochromatosis who are homozygous for the C282Y mutation have a 11-fold increased HCC risk compared to controls (OR = 11, 95%CI: 3.7-34)<sup>[93]</sup>. In a Swedish population-based cohort study, patients with genetic hemochromatosis had a 20-fold increased risk for developing primary liver cancer compared to the general population (standardized incidence ratio: 21, 95%CI: 17-28)<sup>[94]</sup>. In both studies, the presence or absence of cirrhosis was not evaluated. Several other studies reported similar results<sup>[95-98]</sup>.

**Table 1** Characteristics and results of studies on risk of hepatocellular carcinoma in patients with autoimmune hepatitis,  $\alpha$  1-antitrypsin deficiency and Wilson's disease

Ref.	Study design	Study period	Patient No.	Duration follow-up	Results
Autoimmune hepatitis					
Yeoman <i>et al</i> <sup>[110]</sup>	Prospective cohort study	1971-2007	243	median: 11 yr (range 1-36)	Annual HCC incidence: 1.1% HCC occurred more often in cirrhotic patients (9.3% vs 3.4%, $P = 0.048$ )
Wang <i>et al</i> <sup>[111]</sup>	Prospective cohort study	Unknown	124	mean: 111 $\pm$ 6 mo	HCC incidence: 1 per 350 patient-year
Werner <i>et al</i> <sup>[112]</sup>	Retrospective cohort study	1990-2003	473	median: 8.8 yr (range 1-45)	HCC incidence in cirrhotics: 1 per 182 patient-year 23-fold increased HCC risk compared to the general population. Only HCC in cirrhotics
Wong <i>et al</i> <sup>[113]</sup>	Retrospective cohort study	1999-2009	322	mean: 6.25 yr	HCC incidence all patients: 459 per 100000 patient-year (0.5%/yr) In cirrhotics: 1920 per 100000 patient-year (1.9%/yr)
Park <i>et al</i> <sup>[114]</sup>	Retrospective cohort study	Unknown	212 (88 cirrhotics)	mean: 123 $\pm$ 9 mo	HCC incidence in cirrhotics: 1 per 1002 patient-year. (0.1%/yr)
Teufel <i>et al</i> <sup>[115]</sup>	Retrospective cohort study	1970-2009	278 (89 cirrhotics)	mean: 4.8 yr (in cirrhotic pts)	No HCC observed in 431 cirrhotic patient-year
$\alpha$ 1-antitrypsin deficiency					
Eriksson <i>et al</i> <sup>[116]</sup>	Autopsy study	1963-1982	38250 (17 pts with A1AD)	NA	Increased HCC risk in patients with A1AD compared to controls. (OR = 20, 95%CI: 3.5-114.3)
Elzouki <i>et al</i> <sup>[117]</sup>	Autopsy study	1963-1994	50333 (31 pts with A1AD)	NA	Increased HCC risk in patients with A1AD compared to controls (OR = 5.0, 95%CI: 1.6-15.8; $P = 0.008$ ). Only significant in males
Propst <i>et al</i> <sup>[118]</sup>	Retrospective cohort study	1990-1992	Group 1: 240 cirrhotics with different etiologies (25% A1AD) Group 2: 130 non-cirrhotic A1AD pts	Unknown	No significant differences in HCC prevalence between cirrhotic A1AD patients and cirrhotic subjects due to other causes No HCC in non-cirrhotic A1AD patients
Wilson's disease					
Walshe <i>et al</i> <sup>[119]</sup>	Retrospective cohort study	1955-1987 1987-2000 1966-2002	159	range: 10-45 yr	9 patients (6%) developed abdominal malignancies (2 $\times$ HCC). Higher incidence compared to the general population
Thattil <i>et al</i> <sup>[120]</sup>	Case report and review	No limitation	NA	NA	19 published case reports of HCC in patients with Wilson's disease

HCC: Hepatocellular carcinoma; A1AD:  $\alpha$  1-antitrypsin deficiency; NA: Not applicable; pts: Patients.

Primary biliary cirrhosis (PBC) is another important risk factor for HCC development. In a recent meta-analysis, PBC patients had a 19-fold higher risk to develop HCC compared to the general population<sup>[99]</sup>. In a large Japanese cohort of PBC patients, HCC incidence was 2.4% during a mean follow up of 80 mo. HCC incidence was higher in males than in females (5.1% vs 2.0%)<sup>[100]</sup>. Caballeria *et al*<sup>[101]</sup> compared HCC incidence in 140 PBC patients and a group of cirrhotic HCV patients. Cumulative HCC incidence in PBC patients was 3.6% during a mean follow-up of 5.6 years. Incidence rates were much higher (11.1%), when considering only those patients with late stages of disease. PBC patients in stages III and IV appeared to have comparable risks for HCC as cirrhotic HCV patients. Other studies reported similar results<sup>[102-105]</sup>.

The exact incidence of HCC in patients with cirrhosis due to non-alcoholic fatty liver disease (NAFLD) is not known. Nevertheless, a recent systematic review of White *et al*<sup>[106]</sup> reported that patients with NASH-cirrhosis had a consistently higher risk of HCC. Cumulative incidence rates ranged from 2.4% over 7 years to 12.8% over 3 years. In cohorts with non-cirrhotic patients with NAFLD and NASH the risk of developing HCC was

minimal: cumulative HCC mortality ranged from 0%-3% for study periods ranging from 6 to 21 years. Ascha *et al*<sup>[107]</sup> reported that annual cumulative HCC incidence in patients with NASH-cirrhosis was 2.6%, compared to 4.0% in patients with HCV cirrhosis ( $P = 0.09$ ). Besides, in several studies features suggestive of NAFLD were occasionally observed in HCC patients without a well-defined other cause of chronic liver disease<sup>[108,109]</sup>.

Cirrhotics patients with autoimmune hepatitis (AIH)<sup>[110-115]</sup> or  $\alpha$  1-antitrypsin deficiency<sup>[116-118]</sup> seem to have an increased HCC risk. However, the HCC incidence is lower than in cirrhotics with viral hepatitis. HCC in patients with Wilson's disease seems to be rare<sup>[119,120]</sup> (Table 1). Only case-reports are available for HCC in patients with cystic fibrosis<sup>[121-123]</sup>.

### HCC incidence in non-cirrhotic patients

The previously mentioned cost-effectiveness analyses were all restricted to cirrhotic patients and cannot be extrapolated to non-cirrhotic patients. However, also in some subgroups of HBV carriers without cirrhosis, HCC surveillance with US and AFP could be cost-effective<sup>[19]</sup>. HCC incidence should exceed 0.2%/year under these circumstances to allow cost-effective surveillance.

**Table 2 Comparison of recommendations regarding hepatocellular carcinoma surveillance in guidelines**

Characteristics	AASLD guideline <sup>[19]</sup>	EASL guideline <sup>[20]</sup>	APASL guideline <sup>[21]</sup>
Recommended target population	Cirrhotic HBV and HCV patients Alcoholic cirrhosis Stage 4 primary biliary cirrhosis Cirrhosis due to genetic hemochromatosis Cirrhosis due to $\alpha$ 1-antitrypsin deficiency HBV carriers of Asian origin (male > 40 yr, female > 50 yr) African/North American Blacks with hepatitis B HBV carriers with family history of HCC	Cirrhotic patients with Child-Pugh stage A and B Cirrhosis patients with Child-Pugh stage C awaiting liver transplantation Non-cirrhotic HBV carriers with active hepatitis or family history of HCC Non-cirrhotic patients with chronic hepatitis C and advanced liver fibrosis F3	Cirrhotic HBV and HCV patients
Surveillance benefit uncertain	HBV carriers younger than 40 (males) or 50 (females) Hepatitis C and stage 3 fibrosis Non-cirrhotic NAFLD		
Surveillance modality	US	US	US and AFP
Interval (mo)	6	6	6

AASLD: American Association for Study of Liver Disease; APASL: Asian Pacific Association for the Study of the Liver; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; US: Ultrasound; AFP:  $\alpha$ -fetoprotein; HCC: Hepatocellular carcinoma; EASL: European Association for the Study of the Liver.

A prospective population study in 22707 Taiwanese male HBV carriers without cirrhosis showed that annual HCC incidence rate was 0.5%<sup>[124]</sup>. Annual rates increased with age. A Japanese study also reported a relatively high incidence of HCC in HBV carriers (0.4%/year)<sup>[125]</sup>. Both studies only included East Asian patients. In this population, with in general early HBV infection through vertical transmission, annual HCC incidence in non-cirrhotic carriers depends on age and starts to exceed the threshold of 0.2% per year around the age of 40 years<sup>[126]</sup>. In two large community-based prospective cohort studies from Taiwan, predictors of progressive disease in chronic hepatitis B were evaluated<sup>[127,128]</sup>. High transaminases, HBeAg positive status and high serum HBV DNA levels were risk factors for developing HCC. Nevertheless, non-Western HBV carriers remain at significant risk for developing HCC, regardless viral replication status<sup>[127,129,130]</sup>. Although HBsAg loss leads to a pronounced reduction of HCC risk, incidence remains higher than in the general population due to HBV DNA integration in the liver cell<sup>[124,131,132]</sup>.

In contrast, uncontrolled prospective cohort studies in North America have indicated that the HCC incidence in HBV carriers could vary considerably<sup>[133,134]</sup>. Annual HCC incidence rates ranged between 0.06% and 0.46%. Non-Asian HBV carriers without cirrhosis are generally infected in adulthood by horizontal transmission and often exhibit low level of viral replication. They appear to be at limited risk of developing HCC<sup>[135-137]</sup>. However, HBV carriers with HCV or HIV co-infection or with a first degree relative with HCC are at increased risk for developing HCC<sup>[138-140]</sup>. Chronic HBV patients from sub-Saharan Africa often develop HCC at young age<sup>[129,141]</sup>.

The HCC incidence in HCV patients without cirrhosis is not clear. A Japanese study investigated HCC incidence in HCV patients and reported that annual

HCC incidences ranged from 0.5% to 7.9% in untreated chronic HCV patients with mild fibrosis and cirrhosis, respectively<sup>[142]</sup>. However, Lok *et al.*<sup>[82]</sup> reported that annual HCC incidence in HCV patients with bridging fibrosis was only 0.8%. The risk of developing HCC in non-cirrhotic patients with chronic liver disease due to other causes than viral hepatitis, is not exactly known.

## RECOMMENDATIONS OF GUIDELINES

In the last decade, several guidelines for management of HCC have been published worldwide. Most relevant are the practice guidelines of the American Association for Study of Liver Disease (AASLD)<sup>[19]</sup>, the EASL-EORTC Clinical Practice Guidelines on the management of hepatocellular carcinoma<sup>[20]</sup> and the Asian Pacific Association for the Study of the Liver (APASL) consensus recommendations on hepatocellular carcinoma<sup>[21]</sup> (Table 2). The AASLD guideline recommends surveillance for selected groups of cirrhotics (viral hepatitis, alcohol, PBC, genetic hemochromatosis,  $\alpha$ -1 antitrypsin deficiency) and high risk HBV patients without cirrhosis<sup>[19]</sup>. It is stated that there is insufficient evidence to recommend surveillance in HCV patients with advanced fibrosis or non-cirrhotic NAFLD. The EASL guideline advises surveillance in cirrhotics (regardless underlying cause)<sup>[20]</sup>. Also for HCV patients with advanced fibrosis and non-cirrhotic HBV carriers with active hepatitis or family history of HCC, surveillance is recommended. Of note, according to the EASL guideline, the presence of advanced (Child-Pugh C) cirrhosis prevents potentially curative therapies to be employed, and surveillance is not cost-effective under these circumstances, except for patients on the waiting list for transplantation. In fact, Child-Pugh C cirrhosis will also exclude radiologic interventions or sorafenib in palliative setting. According

to the APASL recommendations, cirrhotic patients with HBV and/or HCV should undergo surveillance<sup>[21]</sup>. Regarding modality of surveillance, the AASLD and EASL guidelines both recommend surveillance by US<sup>[19,20]</sup>. According to the APASL guideline, surveillance should be performed by combined US and AFP measurements<sup>[21]</sup>. All three guidelines recommend a surveillance interval of 6 mo<sup>[19,21]</sup>.

## CONCLUSION

In the last decade, there has been a marked increase in therapeutic options for HCC. Nevertheless, curative options are only feasible in case of early detection. Although regular surveillance could be beneficial in this respect, there is only limited evidence for its effectiveness in clinical practice. US at 6-mo intervals appears the most promising tool for surveillance, but the debate on serum tumor markers such as AFP has not yet ended. Defining those high risk subgroups who will benefit most from surveillance remains an important research goal for the near future.

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## Role of stem cells in repair of liver injury: Experimental and clinical benefit of transferred stem cells on liver failure

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### Abstract

Although the liver has a high regenerative capacity, as a result of massive hepatocyte death, liver failure occurs. In addition to liver failure, for acute, chronic and hereditary diseases of the liver, cell transplantation therapies can stimulate regeneration or at least ensure sufficient function until liver transplantation can be performed. The lack of donor organs and the risks of rejection have prompted extensive experimental and clinical research in the field of cellular transplantation. Transplantation of cell lineages involved in liver regeneration, including mature hepatocytes, fetal hepatocytes, fetal liver progenitor cells, fetal stem cells, hepatic progenitor cells, hepatic stem cells, mesenchymal stem cells, hematopoietic stem cells, and peripheral blood and umbilical cord blood stem cells, have been found to be beneficial in the treatment of liver failure. In this article, the results of experimental and clinical cell transplantation trials for liver failure are reviewed, with an emphasis on regeneration.

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**Key words:** Liver regeneration; Liver failure; Stem cell

**Core tip:** Although the liver has a high regenerative

capacity, as a result of massive hepatocyte death, liver failure occurs. In recent years, there has been extensive experimental and clinical research in the field of cellular transplantation. Transplantation of cell lineages involved in liver regeneration, including mature and fetal hepatocytes, fetal liver progenitor and stem cells, hepatic progenitor and stem cells, mesenchymal stem cells, hematopoietic stem cells, and peripheral blood and umbilical cord blood stem cells, have been found to be beneficial for treating of liver failure. Herein, I review the results of experimental and clinical cell transplantation trials for liver failure.

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### INTRODUCTION

The liver provides various vital functions, including protein synthesis, detoxification, bile excretion and storage of vitamins. It is necessary for survival that it should be regenerated following massive damage induced by environmental toxins, infections and alcohol, etc. Although in normal conditions, hepatocytes, the primary cell type of the liver, are in G0 phase of mitosis, following any injury they rapidly enter the G1 phase and undergo mitosis. S-phase hepatocytes can be located in all segments of the lobule in the normal adult liver<sup>[1]</sup>. In the regenerating liver after partial hepatectomy (PH), periportal cells replicate first, probably reflecting their shorter G1 phase<sup>[2]</sup>. The peak of DNA synthesis is within 40-44 h after PH in mice<sup>[3]</sup>. The average life span of the hepatocytes is relatively long, about 5 mo. These long-lived cells are capable of at least 69 cell divisions and can restore normal architecture and impaired function in the injured liver<sup>[4]</sup>. He-

patocytes are the cells that normally shoulder the burden of regenerative growth after liver damage; therefore, they can be considered as the functional stem cells under most circumstances<sup>[5]</sup>.

The liver is the only internal human organ capable of natural regeneration. Detailed studies of the mechanisms that regulate liver growth have been performed in animals subjected to PH or chemical injury. Livers from small animals enlarge after transplantation to reach a liver size in proportion to the size of the recipient animal (e.g., baboons to humans, small dogs to large dogs)<sup>[6]</sup>. In humans, previous studies have shown that the mean liver volume 6 mo after donor hepatectomy was 90.7% of the initial liver volume<sup>[7]</sup>, and that the livers of the right lobe donor group regenerated faster than those of the left lobe donor group<sup>[8]</sup>. In fact, the growth of the liver is a restoration of function; the lobes that are removed do not regrow into their original form<sup>[9]</sup>. Nevertheless, functional restoration may be sufficient for survival of the organism.

The human liver is composed of mainly parenchymal cells, commonly referred to as “hepatocytes”, which are arranged in 1-2 cells-thick plates surrounded by hepatic sinusoids. They constitute 80% of the cell population of the liver. Sinusoidal endothelial cells, perisinusoidal macrophages (Kupffer cells), stellate cells (Ito cells) and liver-specific natural killer cells (pit cells) represent the non-parenchymal cells<sup>[10]</sup>.

Hepatocytes are rich in membranous and non-membranous organelles and inclusions. Bile is secreted into the bile canaliculi, which are a part of the intercellular space isolated by junctional complexes from the rest of the intercellular compartment. Near the portal space, bile canaliculi transform into the canal of Hering, which is lined by both hepatocytes and cholangiocytes<sup>[10]</sup>. The canal of Hering is thought to serve a reservoir of liver progenitor cells. The cell compartment that resides in the canal of Hering has been called the progenitor (in humans) or the oval cell compartment (in rodents)<sup>[11]</sup>. In rodents, the canal barely extends beyond the limiting plate; in contrast, in humans, it extends to the proximate third of the lobule<sup>[12]</sup>. The epithelial cells of the canal, called “oval cells”, are oval in shape and can differentiate into both hepatocytes and cholangiocytes. Thus, it would appear that a name change from oval cells to “hepatic progenitor cells” (HPCs) is required<sup>[13]</sup>. The transdifferentiation of oval cells to hepatocytes may determine survival when it occurs during liver failure in humans.

Adult hepatic stem cells are scarcely detectable under physiological conditions and during the normal process of liver regeneration, presumably because of their small numbers. Analyses of oval cells have raised the possibility that adult hepatic stem cells are present in the canals of Hering, and that oval cells originate from the stem cells and differentiate into both the hepatic and cholangiocytic lineages<sup>[14]</sup>. Kuwahara *et al*<sup>[15]</sup> enumerated four distinct stem cell niches: the canal of Hering (proximal biliary tree), the intralobular bile ducts, the peri-ductal “null”

mononuclear cells and the peri-biliary hepatocytes.

Although the liver has a high regenerative capacity, as a result of massive hepatocyte death, liver failure occurs. Liver transplantation, sometimes the only option for patient survival, often leads to immunological complications. On the other hand, it is limited by the availability of donor organs. In addition to liver failure, for acute, chronic and hereditary diseases of the liver, cell transplantation therapies can stimulate regeneration or at least ensure sufficient function until liver transplantation can be performed. The lack of donor organs and risks of rejection have prompted extensive research in the field of cellular transplantation. In this article, I review hepatic cell types involved in liver regeneration and cell transplantation therapies for liver failure, with an emphasis on regeneration.

## CATEGORIZATION OF STEM CELLS

Stem cells are the main cells of organisms from which all of the mature body cells are derived. Their high proliferative capacity for self-renewal permit them to increase their numbers by symmetric division. They may remain in the undifferentiated state for long periods. When the morphological as well as functional, differentiation begins, these cells differentiate into multiple specialized cell lineages. Stem cells are the source of progenitor cells committed to one or several lineages. The committed progenitor cells exhibit a capacity for active proliferation and supply abundant daughter cells, which in turn give rise to terminally differentiated cells<sup>[14]</sup>.

Stem cells are classified depending on the potential for differentiation into specialized cell types. The most talented stem cells, totipotent cells of the zygote within first 4 d of the intrauterine life, are able to form a full organism in an appropriate microenvironment. However, pluripotent cells, known as “embryonic stem cells” (ESCs), derived from the inner cell mass of the embryo, can form virtually any cell type derived from any of three embryonic germ layers; ectoderm, mesoderm or endoderm. Thus, an embryonic stem cell can form hepatocytes (endodermal in origin), cardiomyocytes (mesodermal in origin), and neurons (ectodermal in origin). Surplus embryos obtained from *in-vitro* fertilization laboratories are the main sources of the ESCs. However, some disadvantages including, high immune reaction risk and ethical concerns, limit their applications. Multipotent stem cells, known as “adult stem cells”, with a relatively limited differentiation potential, can form different cell types of the tissue. These cells reside together with the specialized cell types of the adult tissues and are thought to be responsible for the tissue maintenance and repair. The exact mechanisms that force them to differentiate into a specialized cell type are not fully known. The two major populations of adult stem cells are bone marrow mesenchymal and hematopoietic stem cells (HSCs). Hematopoietic stem cells have a predetermined fate to form all types of mature blood cells. Mesenchymal stem cells

can differentiate into multiple cell lineages, including tendon cells, muscle cells, osteocytes and fat cells. The term “multipotent stromal cell” implies the multipotent stem cells of both bone marrow and of non-marrow tissue, such as umbilical cord blood, adipose tissue, muscle tissue and dental pulp. In laboratory conditions, multipotent cells show plasticity. “Plasticity” or “transdifferentiation” means that the stem cells of an adult tissue can generate differentiated cells types of a different tissue. For instance, HSCs can transform into hepatocytes or brain stem cells or form skeletal muscle fibers. It is not clear if this occurs in the body. Multipotent cells do not cause any immune reaction, because they are genetically identical to their hosts. However, these cells are restricted in their ability to form different cell types. Moreover, they have some disadvantages, including slow rate of cell division and difficulties in isolating sufficient numbers for application because of their scarcity within tissues. The last type of stem cells is unipotent stem cells, which have very limited capacity for differentiation and can give rise to only one type of cell under normal conditions. For example, unipotent stem cells of colony forming unit of erythrocytes (CFU-E) can only give rise to mature blood erythrocytes.

In recent years, stem cells have been widely studied for their potential therapeutic use. However, some of studies were not successful. Researchers agree that as well as isolation of adequate numbers of healthy stem cells, selection of the most convenient transportation route, regulation of stem cell differentiation into a special cell type and obtaining the normal functions of the differentiated cells are very important regarding the benefit of stem cell applications. The most important risk of the transplanted stem cells is generation of tumors if cell division occurs in an uncontrolled manner. Unfortunately, stem cell transplantation therapy may be considered as a double-edged sword.

## HEPATIC CELLS INVOLVED IN REGENERATION

The liver can regenerate itself by increasing the rate of hepatocyte mitosis and differentiation of stem cells into hepatocytes or cholangiocytes. Stem cells are the main cell lineage for liver regeneration. Several studies suggest the existence of one or more population of cells (*e.g.*, stem cells, progenitor cells and extrahepatic stem cells) that are able to differentiate into hepatocytes and biliary epithelial cells. However, the exact location of these cells is not yet clear. In humans and rodents, potential liver stem cells may exist within the biliary tree. Both rodent and human ESCs, bone marrow HSCs, mesenchymal stem cells (MSCs), umbilical cord stem cells, fetal and adult liver progenitor cells, and mature hepatocytes have been reported to be capable of self-renewal, giving rise to daughter hepatocytes both *in vivo* and *in vitro*<sup>[16]</sup>. Although the factors controlling proliferation, differentiation and secretion processes are not well defined, recent studies

emphasize the role of several local (microenvironment) and systemic factors. However, the exact triggering mechanisms for differentiation of these cells into mature hepatocytes are not fully understood.

During embryonic development, hepatoblasts generate the two epithelial cell lineages: hepatocytes and biliary cells<sup>[17]</sup>. The area connecting the terminal segment of the biliary ductular system with parenchymal hepatocytes persists in the adult liver and is known as the canals of Hering<sup>[18]</sup>. The primitive intrahepatic bile ducts expressing both hepatocyte proteins and biliary epithelial markers have consequently been referred to as “transitional cells”<sup>[19-21]</sup>. Transitional cells have properties intermediate between those of oval cells and hepatocytes<sup>[20]</sup>. These cells are believed to remain in the adult liver as bipotential progenitors for both hepatocytes and biliary cells<sup>[21]</sup>.

Many investigators favor the view that the liver harbors facultative stem cells that are located throughout the biliary epithelium. The activation of these cells for transformation into mature hepatocytes is a conditional process that occurs only when the regenerative capacity of hepatocytes is overwhelmed<sup>[22]</sup>. Hepatocyte differentiation within bile ducts in the human liver has been noted, which has led to the belief that small biliary cells, hepatocyte-like cells expressing both markers of bile duct cells and hepatocytes, which repopulate severely damaged liver parenchyma, can function as a progenitor cell population for new hepatocytes<sup>[23]</sup>. In rodents, early reactive bile ductules do not generally resemble hepatocytes, but later acquire features of hepatocytes<sup>[22]</sup>. By contrast, direct evidence for the transformation of hepatocytes into biliary cells provided in cell culture had raised a possibility that hepatocytes themselves may be precursor cells for the biliary epithelium if the latter’s ability to proliferate and repair themselves is compromised for some reason<sup>[24,25]</sup>.

The oval cells represent the progeny of liver stem cells and function as an amplification compartment for the generation of “new” hepatocytes<sup>[22]</sup>. The oval cell compartment, consisting of small ovoid cells with scant, lightly basophilic cytoplasm and pale blue staining oval nuclei<sup>[26]</sup>, is widely used to describe liver progenitors. It is generally accepted that oval cells are bipotential transit-amplified cells derived from normally quiescent “true stem cells”, which reside in the biliary tree and are absent in the healthy liver<sup>[27]</sup>. In fact, to date, whether oval cells pre-exist in the tissue or develop from other adult cell types (*e.g.*, bile duct cells) after injury, is unknown. The restricted potential to differentiate into hepatocytes and cholangiocytes qualifies oval cells more as progenitor cells than as true stem cells<sup>[28]</sup>. The oval cells compartment can probably not to be attributed to a single cell type. A primitive oval cell population that do not express alpha-fetoprotein (AFP), cytokeratin 19 (CK-19), OV-6; a hepatocyte-like oval cell population that express AFP, but not OV-6; and a ductular-like oval cell population that not express AFP, but express CK 19 and OV-6 have been isolated<sup>[29]</sup>. It is presently unclear if antigenically distinct subpopulations of oval cells are derived from different

precursor cells or if their phenotype merely reflects the commitment of an oval cell to a specific lineage<sup>[30]</sup>.

Oval cells form ductular structures that communicate with the biliary system at one end and terminate at a hepatocyte-forming blind end<sup>[31]</sup>. Markers commonly used to assess differentiation and to trace lineages of oval cells include expressed antigenic markers for hepatocytes, biliary ducts and oval cells (BSD7, OC2, OC3, OV-1, and OV-6), intermediate filaments, extracellular matrix proteins (CK8, 18, 19), enzymes and secreted proteins (alpha-fetoprotein and gamma-glutamyl transferase)<sup>[32,33]</sup>. Oval cells also express some markers considered characteristic of stem cells, including stem cell factor<sup>[34]</sup>, bcl-2<sup>[35]</sup> and cytokeratin 14<sup>[36]</sup>. They are also immunoreactive to antibodies generally associated with hematopoietic lineages, such as CD34, and c-kit<sup>[37,38]</sup>; therefore, there may be a common lineage between hematopoietic and liver cell precursors. In a recent study, a population of cells (beta-2-microglobulin-ve, Thy-1+ve) in rat and human bone marrow was identified that also expressed hepatocyte specific functions, suggesting that these cells may be hepatic stem cells. After intraportal infusion into rat livers, rat-derived bone marrow cells integrated with hepatic cell plates and differentiated into mature hepatocytes<sup>[39]</sup>. Moreover, Crosby *et al*<sup>[37]</sup> have shown that *c-kit* and CD34 positive cells isolated from human liver are able to differentiate into biliary epithelial cells and endothelial cells. Thus, biliary cells and endothelial cells may also share some common precursors. It has been postulated that oval cells arise either from cells lining the canals of Hering<sup>[31,40]</sup>, from mature biliary cells<sup>[12]</sup>, liver epithelial or stromal cells<sup>[41]</sup>, or from circulating hematopoietic stem cells<sup>[42,43]</sup>. Additionally, some antigens traditionally associated with hematopoietic cells (c-kit and CD34) can also be expressed by oval cells, leading to the notion that at least some hepatic oval cells are directly derived from a precursor of bone marrow origin<sup>[39,44]</sup>. Fausto *et al*<sup>[45]</sup> suggested that bone marrow stem cells can generate oval cells and hepatocytes; however, transdifferentiation is very rare and inefficient. Bone marrow derived hepatocytes constituted from 0.008% to 0.8% of total parenchymal cells; therefore, differentiation of bone marrow cells into mature hepatocytes is very inefficient under physiological conditions<sup>[46]</sup>. Additionally, the repopulation process is not complete and is relatively slow<sup>[43,47]</sup>.

Studies have demonstrated that HSCs have the capacity to fuse with other cell types<sup>[48]</sup>. Several publications subsequently emerged to demonstrate that the appearance of new hepatocytes from bone marrow precursors in liver repopulation models was not caused by transdifferentiation of the marrow stem cells to hepatocytes, but to fusion of the marrow cells with the hepatocytes of the recipient<sup>[48,49]</sup>. While fusion with hepatocytes in whole animal experiments may have a role, it cannot explain the appearance of hepatocyte-like cells in cell cultures of bone marrow<sup>[25]</sup>.

The periductular stem cells are one of the other cell types related to liver regeneration in some types of liver

injury. These cells are rare in the liver, have a very long proliferation potential, and may be multipotent; however, their full potential has yet to be defined. These cells may be hematopoietic stem cell types that either reside in liver or bone marrow<sup>[50]</sup>.

Another cell type related to the regeneration of rat liver has been identified, referred to as "small hepatocytes" (small hepatocyte-like progenitor cells)<sup>[51,52]</sup>. This cell population is phenotypically different from fully differentiated hepatocytes, cholangiocytes and oval cells. They represent a unique parenchymal (less differentiated) progenitor cell population<sup>[53]</sup>. These cells have an extensive proliferative capacity and may represent a novel progenitor cell population that responds to liver deficit when the replicative capacity of differentiated hepatocytes is impaired, and can restore tissue mass<sup>[52]</sup>. However, there is still controversy as to whether these cells represent an intermediate state in oval cell differentiation or are derived from hepatocytes resistant to stem cells. Best *et al*<sup>[54]</sup> suggested that small hepatocytes are not the progeny of oval cell precursors, but represent an independent liver progenitor cell population. By contrast, Vig *et al*<sup>[55]</sup> showed that oval cells can form small hepatocyte-like progenitor cell nodules during the regeneration stage after chronic hepatocellular liver injury.

A number of studies have been published demonstrating that stem/progenitor cells can be differentiated toward "hepatocyte-like cells", a term that has been used to describe cells generated *in vitro* that show some characteristics of mature hepatocytes, but are still not fully mature and/or characterized<sup>[56]</sup>. Classic studies by Evarts *et al*<sup>[57-59]</sup> demonstrated that oval cells gradually transform themselves into small basophilic hepatocytes, which then become fully mature hepatocytes and replace the lost liver mass. They also showed the transfer of radiolabeled thymidine from oval cells to newly formed hepatocytes *in vivo*. Thus, the precursor-product relationship between oval cells and basophilic hepatocytes has been suggested<sup>[59]</sup>.

Recently, a unique population of liver-derived bipotential liver progenitors was isolated from unmanipulated rat liver<sup>[60]</sup>. These bipotential liver cells express both hematopoietic stem cell markers, such as CD45, CD34 and thy-1, similar to oval cells<sup>[60,61]</sup>, and endodermal/hepatic markers. In contrast to oval cells, these liver progenitors are negative for OV-6, cytokeratin 7 and CK 19, and express very little or no AFP<sup>[60,62]</sup>. Their capacity for hepatic differentiation makes them a valuable resource for important applications such as cell therapies for a variety of liver diseases<sup>[62]</sup>.

Although mature hepatocytes and cholangiocytes represent the first and most important resource for tissue repair, experimental data support the hypothesis that the liver also contains or activates a stem cell compartment<sup>[63,64]</sup>. Herrera *et al*<sup>[65]</sup> isolated a pluripotent population similar to rodent oval cells from adult liver and may be more mesenchymal in lineage. These cells expressed mesenchymal stem cell markers, but not the hematopoi-

etic stem cell markers. The absence of staining for cytokeratin-19, CD117, and CD34 indicated that these cells were not oval stem cells.

Castorina *et al*<sup>[64]</sup> reported that human liver stem cells express several mesenchymal markers, such as CD 44, but not hematopoietic stem cell markers. Additionally these multipotent cells express AFP, albumin, CK7 and CK19, indicating a partial commitment to hepatic and biliary lineages. Schmelzer *et al*<sup>[66,67]</sup> isolated two pluripotent hepatic progenitors: hepatic stem cells and progenitors. The gene expression profile of hepatic stem cells throughout life consists of high levels of expression of cytokeratin 19 (CK19), neuronal cell adhesion molecule (NCAM), epithelial cell adhesion molecule (EpcAM), and claudin-3 (CLDN-3); low levels of albumin; and a complete absence of expression of AFP. By contrast, hepatoblasts, found as < 0.1% of normal adult livers, express high levels of AFP, elevated levels of albumin, low levels of CK19 and a loss of NCAM and CLDN-3.

Notably, both hepatocytes and hepatic progenitor cells may differentiate into hepatocytes and biliary cells, as well indicating their bipotent differentiation capacity. Hence, both cell types meet the minimal definition criteria of a stem cell, *i.e.*, the potential of self-renewal to maintain the stem cell reserve, and a multiple differentiation potential giving rise to progeny of at least two different lineages<sup>[68]</sup>.

## FACTORS RELATED TO HEPATIC REGENERATION

Studies of liver injury have led the identification of several factors that are involved in the regulation of cell activation related to liver regeneration. It is not clear whether the same factors known to be involved in normal hepatic regeneration are also involved in regeneration *via* the stem cell compartment.

As mentioned before, the hepatic progenitor cell niche is located at the level of the canals of Hering. The ductular and periductular area is composed of numerous different cells, such as portal myofibroblasts, stellate cells, endothelial cells, hepatocytes, cholangiocytes, Kupffer cells, pit cells and inflammatory cells. All these cells could interact and crosstalk with hepatic parenchymal cells, influencing their proliferative and differentiative processes through the provision of numerous signals within the niche<sup>[5]</sup>. The local environments of endogenous and transplanted cells mainly affect their proliferation, differentiation, secretion, and other functions<sup>[69,70]</sup>. Hepatocyte growth factor (HGF), epidermal growth factor (EGF), and transforming growth factor- $\alpha$  (TGF- $\alpha$ ), as potent mitogens, are primarily associated with normal hepatic regeneration<sup>[71-73]</sup>. In cultures, the mouse liver progenitor cells differentiated into hepatocytes upon treatment with EGF or differentiated into biliary lineage cells upon treatment with HGF<sup>[74]</sup>. In their quiescent state, hepatocytes do not fully respond to growth factors such as HGF, TGF and EGF, which are potent stimulators of DNA

replication for hepatocytes in primary culture<sup>[75-77]</sup>. In the intact liver, hepatocytes need to be “primed” to enter the cell cycle and respond to growth factors<sup>[73]</sup>. The results show that TNF acts as a primer to sensitize hepatocytes to the proliferative effects of growth factors, and offers a mechanism to explain the initiation and progression phases of liver regeneration after PH<sup>[77]</sup>.

In addition to hepatocyte-autonomous signals, endocrine and paracrine factors are critical to normal regeneration, and extensive work has focused on the role of the liver microenvironment, *i.e.*, non-parenchymal cells and the extra-cellular matrix (ECM), in liver homeostasis and regeneration<sup>[71,78]</sup>. Non-parenchymal cells, such as endothelial cells, Kupffer cells, stellate cell and intrahepatic lymphocytes provide critical signals to hepatocytes during regeneration<sup>[78-80]</sup>. Intercellular interaction seems to be crucial during liver regeneration. Indeed, the initiation of liver regeneration involves the rapid and simultaneous activation of multiple signaling pathways in both hepatocytes and non-parenchymal cells, which are the main sources of tumor necrosis factor, interleukin-6, and heparin binding EGF<sup>[75,76,81]</sup>. Following acute liver injury, release of IL-6 from Kupffer cells and neutrophils and the growth factors including HGF, EGF, TGF- $\alpha$ , and fibroblast growth factor- $\alpha$  released from hepatic stellate cells, stimulate hepatocytes to enter mitosis<sup>[76,81]</sup>. Stellate cells are regarded as the principal source of extracellular matrix proteins during hepatic regeneration<sup>[82]</sup>. A recent study demonstrated that HSCs act as a positive regulator at the early phase and a negative regulator at the terminal phase of liver regeneration through cell-cell interaction and cytokine networks<sup>[83]</sup>. The authors reported that high levels of HGF at early phase of liver regeneration stimulated oval cell proliferation *via* extracellular signal-regulated kinase and p38 pathway, whereas high levels of TGF- $\beta$ 1 at the terminal phase of liver regeneration suppressed DNA synthesis of oval cells. The shift between these two distinct effects depended on the balance between HGF and TGF- $\beta$ 1 secreted by HSCs. Paku *et al*<sup>[31]</sup> demonstrated that proliferating oval cells are closely associated with stellate cells, suggesting that non-parenchymal cells nurture oval cell growth and differentiation through secretion of growth factors and cytokines, and also by direct cell-to-cell interactions. The factors involved in the regulation of oval cell activation include TGF, HGF and its receptor c-met, IL-6 and peroxisome proliferators/peroxisome proliferator activated receptor alpha<sup>[84-87]</sup>. It is clear that from the first stem/progenitor activation phase to the final differentiation phase of the oval cell cycle, several growth factors and other factors are effective.

More recent studies have emphasized the involvement of TNF-like weak inducer of apoptosis (TWEAK), a member of the TNF family, in the proliferation of oval cells. TWEAK expressed by T cells can stimulate hepatic progenitor cell proliferation. It appears that TWEAK selectively promotes proliferation of oval cells without having an effect on hepatocytes<sup>[87]</sup>.

Some of the other key molecules in the liver microen-

environment that determine regenerative behavior include the pro-inflammatory cytokines and angiogenic factors, such as vascular endothelial growth factor (VEGF)<sup>[80,88]</sup>.

Changes in microenvironments may have contributed to the positive outcomes of many liver cell transplantation studies, and might be initiated by the strong outputs (*e.g.*, signaling, secretion) from the transplanted hepatocytes that drastically affect the environments to stimulate endogenous hepatocyte regeneration<sup>[89]</sup>. Improvement of liver microenvironments related to liver regeneration is one of the goals of cell transplantation therapies. Recently, numerous experimental and clinical studies have been performed investigating the factors that increase the benefit of cell transplantation therapies and survival of the patients with liver damage or failure.

## CELL TYPES TRANSPLANTED FOR LIVER FAILURE

Cell transplantation therapy is a promising alternative approach that leads to donor cell-mediated repopulation of the liver and improved survival rates in experimental models of liver disease. It may serve to alleviate the symptoms while the patients are waiting for liver transplantation. However, significant challenges remain before these cells can be used in humans, such as the lack of consensus about the immunophenotype of liver progenitor cells, uncertainty of the physiological role of reported candidate stem/progenitor cells, practicality of obtaining sufficient quantity of cells for clinical use, and concerns over ethics, long-term efficacy, and safety<sup>[16]</sup>. A registered clinical application based on stem cell technology will take at least an additional 5-10 years because of certain limitations; *e.g.*, the lack of suitable cell sources and risk of teratoma formation<sup>[90]</sup>.

Stem cell therapy exerts its beneficial effect through a number of mechanisms, not necessarily transdifferentiation. Paracrine factors also have an important role in the improvement mechanism. Mature hepatocytes, stem/progenitor cells (ESCs, adipose-derived stem cells, umbilical stem cells, bone marrow-derived stem cells and oval cells), and hepatocyte-like cells are the main cell types used for cell transplantation in experimental and/or clinical studies. Transplanted hepatocytes have high function, but short survival time, whereas transplanted stem/progenitor cells have weak function, but high proliferative capacity. Hepatocyte-like cells accumulate over time *via* differentiation and proliferation<sup>[91]</sup>. However, the numbers of hepatocytes needed for transplantation in humans can be quite large<sup>[92]</sup>, cells that can differentiate into mature hepatocytes have been great interest. Additionally, since hepatocytes are large in diameter, up to 70% of transplanted hepatocytes get trapped in the hepatic sinusoids, which leads to temporary obstruction with subsequent portal hypertension<sup>[93]</sup>, and they have a poor engraftment rate<sup>[94]</sup>.

## MATURE HEPATOCYTES

Hepatocyte transplantation has been performed for more than 10 years in humans, meeting with varied degrees of success<sup>[95]</sup>. Data published for almost 70 years have unequivocally shown that hepatocytes are the replicating cells responsible for liver regeneration and that progenitor cell activation leading to lineage generation is not observed during this process<sup>[3,19,96]</sup>. Although the other cell types of the liver are necessary to support hepatocyte replication and hepatic growth, it has now been established that the hepatocyte has a remarkable capacity for cell proliferation and is the most efficient cell for liver repopulation after injury<sup>[45,75]</sup>. Therefore, transplantation of mature hepatocytes into an injured liver seems to be helpful to support recovery process. However, transplanted hepatocytes have a low liver-engraftment rate and survival<sup>[97]</sup>, and hepatocytes are only available from cadaveric donor livers, which mean that the cells largely lack transplantation quality and quantity. Moreover, cryopreservation of mature hepatocytes before use leads to an additional substantial loss of viability and function. Thus, research is aiming to obtain transplantable cells from embryonic and adult stem cells, or liver progenitor cells that can be expanded *in vitro*. One attractive alternative source of transplantable hepatocytes is cells derived from an immortalized hepatocyte cell line that provides an unlimited supply of transplantable cells<sup>[98]</sup>. Immortalized hepatocytes could then grow in tissue culture and subsequently function as differentiated, non transformed hepatocytes following transplantation<sup>[98,99]</sup>.

### Experimental results

Rhim *et al*<sup>[100,101]</sup> showed that a small number of transplanted hepatocytes could repopulate the liver of newborn urokinase-type plasminogen activator (uPA) transgenic mice. Transplantation of rat liver cells into these mice resulted in the complete reconstitution of a mouse liver with rat hepatocytes. The transplanted liver cell populations replaced up to 80 % of the diseased recipient liver. Overturf *et al*<sup>[102]</sup> found evidence that short-term therapeutic liver repopulation does not require progenitor or stem cells. The majority of the transplanted cells apparently participated in the repopulation process and intermediate-size hepatocytes appeared to have a better replicative capacity than small hepatocytes. Recently, transplanted hepatocytes were shown to engraft in the liver of animals with acute liver failure (ALF)<sup>[103]</sup>. However, only 20%-30% of the transplanted hepatocytes survive and engraft in the liver of rats<sup>[104]</sup>. In fact, several studies using rat models of primary hepatocyte transplantation revealed that transplantation leads to efficacious donor chimerism<sup>[105-107]</sup>. When hepatocytes were transplanted *via* the spleen, cells were distributed immediately in periportal areas, fibrous septa and regenerative nodules of the cirrhotic liver<sup>[107]</sup>. However, transplanted cell proliferation in the liver was limited, and animals did not show any dif-

ferences in mortality over a 12-mo period. On the contrary, Kobayashi *et al*<sup>[108]</sup> found that intrasplenic cell transplantation in extremely sick cirrhotic rats was associated with improvement in liver tests, coagulation abnormality and outcomes. Additionally, cell transplantation has been shown to prevent the development of intracranial hypertension in pigs following acute ischemic liver failure<sup>[109]</sup>.

Immortalized hepatocytes have also been shown to improve the survival rate in an ALF model<sup>[110]</sup>. Immortalized hepatocytes that can function as well as primary hepatocytes following transplantation were found to be effective in the treatment of liver failure in rats with end-stage cirrhosis with hepatic encephalopathy<sup>[98,111]</sup>. The immortalized hepatocytes may achieve a meaningful liver population using a clonal cell line; however, the malignant potential of these immortalized cell lines needs to be fully investigated before they could be applied in the clinic.

### Clinical results

In an early study, in 10 Japanese patients with cirrhosis, hepatocytes ( $1-60 \times 10^7$ ) isolated from a piece of their own liver were transplanted into various sites, including the spleen<sup>[112]</sup>. In one of these patients, transplanted hepatocytes were detected in the spleen 11 mo following transplantation. One of these patients recovered. In another trial, five patients with hepatic encephalopathy and multiple organ failure were transplanted with allogeneic hepatocytes ( $2.8 \times 10^7-2.9 \times 10^7$ ) through the splenic artery<sup>[113]</sup>. Biochemical evidence of liver injury improved significantly and blood ammonia levels decreased significantly to normal levels in the hepatocyte-treated patients. Three of these patients bridged to liver transplantation and were normal with more than 20 mo of follow-up. Transplantation of hepatocytes *via* the abdominal cavity also has been found beneficial. Seven patients with fulminant hepatic failure (FHF) were transplanted ( $6 \times 10^7/\text{kgBW}$ ) *via* the abdominal cavity resulting in survival and improved encephalopathy<sup>[114]</sup>.

Cryopreserved hepatocyte transplantation is a bridging method while patients with chronic liver failure await liver transplantation. Three of five patients with ALF who received transplantation of  $1.3 \times 10^9-3.9 \times 10^{10}$  cryopreserved hepatocytes through intrasplenic and intraportal infusion improved afterwards<sup>[115]</sup>. A patient with ALF infused intraportally with  $8 \times 10^9$  cryopreserved human hepatocytes fully recovered 12 wk after transplantation<sup>[116]</sup>. Repeated application of primary human hepatocytes seems to be safe and results in measurable benefits for patients with ALF.

## HEPATIC PROGENITOR/STEM CELLS

Human hepatic stem cells, constituting approximately 0.5%-2.5% of liver parenchyma, can be isolated by immunoselection for epithelial cell adhesion molecule-positive cells (EpCAM+)<sup>[67]</sup>. Isolation of hepatic progenitor cells from human material has proven to be very difficult. In fact, although hepatic progenitor cells express

several markers, their unequivocal isolation as a pure fraction has been a major obstacle in liver progenitor cell research. Novel cell surface markers in adult progenitor cells include tight junction proteins, integrins, cadherins, cell adhesion molecules, receptors, membrane channels and other transmembrane proteins. Cell surface markers, CD133, claudin-7, cadherin 22, mucin-1, ros-1 and Gabrp 9 are overexpressed and are unique for the adult progenitors<sup>[117]</sup>. Thymus cell antigen 1 (Thy-1) is a marker for sorting bipotential progenitor cells from human livers<sup>[118]</sup>. None of the described markers are completely specific; therefore, isolation of viable cells is limited<sup>[119]</sup>.

Much less is known about the mechanisms of oval cell replication and differentiation, although new information on these topics is rapidly accumulating. Regarding cellular aspects of liver growth and regeneration, it needs to be established what kind of signaling mechanisms may exist, direct and/or indirect, between hepatocytes and oval cells that determines whether one cell type or the other is the main or initial target for a growth stimulus<sup>[45]</sup>.

### Experimental results

Schmelzer *et al*<sup>[67]</sup> demonstrated that purified EpCAM+ cells from fetal or postnatal livers are able to engraft the livers of immunodeficient adult mice (with or without prior injury) and give rise to mature human liver parenchymal cells. Similar results were obtained by Weiss *et al*<sup>[118]</sup> through the isolation of Thy-1+ cells from adult human livers and their transplantation in immunodeficient Pfp/Rag2 mice. Analysis of *in situ* material revealed that transplanted cells express human hepatic markers HepPar1 and albumin, indicating functional engraftment.

Oval cell proliferation is prominent in many models of liver injury, including CCl<sub>4</sub> treatment in combination with PH<sup>[120,121]</sup>. A recent study showed that transfer of oval cells to Wistar rats with FHF could significantly increase their survival rate<sup>[122]</sup>. In the study of Wang *et al*<sup>[123]</sup>, 3,5-diethoxycarbonyl-1,4-dihydrocollidine induced oval cell proliferation. Transplantation of murine oval cells could repopulate the recipient liver in fumarylacetoacetate hydrolase-deficient mice, and rescue the phenotype.

### Clinical results

As far as I know, to date, no clinical application has been performed.

## FETAL HEPATOCYTES/FETAL LIVER PROGENITOR CELLS/FETAL STEM CELLS

Fetal human hepatocytes exhibit unique properties, including the capacity for extensive proliferation and excellent recovery following partial liver resection<sup>[124]</sup>. However, experimental studies have predominantly focused on transplantation of fetal hepatic progenitor cells. Oertel *et al*<sup>[125]</sup> purified hepatic stem/progenitor cells from fetal livers that are fully capable of repopulating the normal adult liver. This represents a major advance toward developing

protocols that will be essential for clinical application of liver cell transplantation therapy.

### Experimental results

After transplantation of mouse fetal liver progenitor cells into 14 to 20 d-old uPA-mice with subacute liver failure, donor-derived regeneration nodules were detectable. Fetal liver cells showed a mature hepatic phenotype, as established by gene expression profiling and a functional integration within in the first 4 wk after transplantation<sup>[126]</sup>. Transplanted rat fetal liver epithelial progenitor cells were able to repopulate a recipient liver subjected to PH, alone or with retrorsine, in syngeneic dipeptidyl peptidase IV (DPPIV) mutant rats<sup>[127]</sup>. Progenitor cells were able to differentiate into both hepatocytes and bile epithelial cells, unlike mature hepatocytes that are not able to differentiate to bile epithelial cells. Moreover, progenitor cells continued to proliferate for longer than hepatocytes after transplantation. Likewise, Dlk+ hepatic stem/progenitor cells purified from rat midgestational fetal livers were able to extensively repopulate the host liver in syngeneic DPPIV mutant rats subjected to PH alone<sup>[125]</sup>. In the CCl<sub>4</sub> rat model of FHF with 2/3 hepatectomy, fetal liver stem/progenitor cells were found to be effective to repair the damaged liver<sup>[89]</sup>. Thus, fetal hepatic stem/progenitor cells exhibit potency for reconstitution of the adult liver under a particular set of conditions.

### Clinical results

As far as I know, to date, no clinical application has been performed.

## EMBRYONIC STEM CELLS

### Experimental results

The first report of hepatic differentiation of mouse embryonic cells was in 2001 by Hamazaki *et al*<sup>[128]</sup>, who produced an embryoid body from an ES cell and subsequently added fibroblast growth factor, HGF, oncostatin M (OsM) and dexamethasone (Dex) to induce the differentiation of cells exhibiting hepatocyte-like properties. The results of Heo *et al*<sup>[129]</sup> are particularly noteworthy, as they report that liver precursor cells induced from ES cells in the absence of exogenous growth factors or feeder cell layers also have the ability to differentiate into biliary epithelial cells. In 2003, Yamamoto *et al*<sup>[130]</sup> produced hepatic cells with a high level of liver function by transplanting ES cells into mice livers 24 h after CCl<sub>4</sub> intoxication. In terms of ultrastructural analysis, these ES-derived hepatocytes were generally similar to normal hepatocytes. Additionally, no teratoma formation was observed in the transplant recipients. In the study of Hu *et al*<sup>[131]</sup>, ES-derived hepatocytes could improve the life quality and lengthen the survival time of CCl<sub>4</sub>-induced FHF. Sprague-Dawley rats with surgically induced liver failure *via* 90% hepatectomy, receiving 10<sup>6</sup>-10<sup>8</sup> ESCs as splenic transplantation, showed 100% survival rate up to 3 mo<sup>[132]</sup>. Similarly, hepatocytes derived from ES cells in

a bioartificial assisted liver device were able to improve survival in rats with liver failure induced by galactosamine after 10 h of extracorporeal liver dialysis<sup>[133]</sup>. Additionally, embryonic derived hepatocytes, implanted subcutaneously as a bioartificial liver device into mice subjected to 90% hepatectomy, reversed the liver failure<sup>[134]</sup>. Transplantation of ES cell-derived hepatic cells significantly suppressed the onset of fibrosis in mice<sup>[135]</sup>.

### Clinical results

Embryonic stem cell studies remain at the preclinical stage because of the risk of teratomas. Despite these successful animal studies, there have been no clinical trials using human ES cells to treat liver diseases in human patients, because utilization of human ES cells raises serious ethical questions in many countries.

## MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are an adult stem cells population found in numerous living tissues. It has been reported that among MSCs obtained from bone marrow, adipose tissue, umbilical cord blood and placenta, several hepatocyte-like cells have the ability to differentiate<sup>[136-138]</sup>. Besides, MSCs, immune-privileged cells with low MHC I and no MHC II expression have low rejection risk and, as such, are a particularly promising source of cells for the treatment of acute and degenerative liver diseases<sup>[136]</sup>. Chamberlain *et al*<sup>[139]</sup> transplanted clonal human MSCs into preimmune fetal sheep by intrahepatic and intraperitoneal routes in their study. The intrahepatic injection of human MSCs was safe and resulted in more efficient generation of hepatocytes throughout the liver parenchyma at days 56-70. Human MSCs cells accumulated in the injured liver. The injured liver may produce regulatory factors for homing of stem cells to the injury site<sup>[135]</sup>.

### Bone marrow-derived mesenchymal stem cells

The most important source of MSCs is bone marrow.

**Experimental results:** A recent study by Carvalho *et al*<sup>[140]</sup> demonstrated that MSCs injection into the portal vein of mice or rats with liver cirrhosis induced by CCl<sub>4</sub> and ethanol did not reduce hepatic fibrosis or promote any improvement in parameters of liver function. However; Oyagi *et al*<sup>[141]</sup> demonstrated benefits in transplantation of bone marrow-derived mesenchymal stem cells (BMMCs) cultured with HGF in CCl<sub>4</sub>-induced rats. Transplantation of the BMMCs into liver-injured rats restored their serum albumin level and significantly suppressed transaminase activity and liver fibrosis. These effects were not seen when the BMMCs were cultured without HGF. Similar results of Fang *et al*<sup>[142]</sup> supported the beneficial effects of BMMCs on reducing collagen deposition.

**Clinical results:** Autologous BMMC transplantation to 53 patients with liver failure caused by hepatitis B had fa-

avorable short-term efficacy with improved levels total bilirubin, prothrombin time and Model for End-Stage Liver Disease score of patients 2-3 wk after transplantation<sup>[143]</sup>. Patients who received 120 mL of autologous bone marrow fluid *via* a hepatic artery showed improved hepatic function in the early period (1-48 wk). Analysis showed no adverse effects from bone marrow administration a long observation period. Additionally, data obtained from eight patients with liver cirrhosis showed that MSCs injection through peripheral or portal vein under ultrasound guidance could be used for the treatment of end-stage liver disease with satisfactory tolerability<sup>[144]</sup>. The study of Amer *et al*<sup>[145]</sup> reported the safety and short-term efficacy of autologous bone marrow-derived hepatocyte-like cell transplantation in the treatment of patients with end-stage liver cell failure. Comparing hepatic and splenic routes of injection, there was no significant difference, except in the first month. The splenic route was technically easier, although it was associated with a higher incidence of mild complications (fever and transient shivering).

#### **Placenta-derived mesenchymal stem cells**

Another promising source of MSCs is the placenta. Human placental MSCs are free of ethical concerns, are non-invasively accessible, abundant and strongly immunosuppressive<sup>[146,147]</sup>. Placenta derived MSCs can be differentiated into hepatocyte-like cells *in vitro*<sup>[148]</sup>.

**Experimental results:** The experimental study of Cao *et al*<sup>[149]</sup> revealed that human placental MSCs could not only differentiate into hepatocyte-like cells *in vitro* and *in vivo*, but also could prolong the survival time of pigs with ALF. The survival rate was significantly higher in the transplantation group than in the control group (66.7% *vs* 0%). Recently, van Poll *et al*<sup>[150]</sup> provided evidence that MSC-derived molecules directly inhibit hepatocellular death, enhance liver regeneration and ultimately improve survival in rats undergoing D-galactosamine-induced FHF. Systemic infusion of MSC-conditioned medium resulted in a 90% reduction of apoptotic hepatocellular death and a three-fold increment in the number of proliferating hepatocytes. Moreover, transplanted human placental MSCs ameliorate CCl<sub>4</sub>- induced liver cirrhosis by their anti-fibrotic effect in a rat model<sup>[151]</sup>. Mohsin *et al*<sup>[152]</sup> reported that pretreated MSCs expressing high levels of albumin, cytokeratin 8, 18, TAT and HNF1 $\alpha$  transplanted in the left lateral lobe of mice with liver fibrosis resulted in a significant reduction in the fibrotic area in the liver, concomitant with improved serum levels of bilirubin and alkaline phosphatase. Cao *et al*<sup>[149]</sup> compared the effects of transplantation of placental MSCs through the peripheral (jugular) and portal veins; their data suggested that both transplantation routes were safe, with no portal vein thrombosis. However, histological data revealed that transplantation of human placental MSCs *via* the portal vein reduced liver inflammation, decreased hepatic denaturation and necrosis, and promoted liver regeneration.

**Clinical results:** Despite the several positive results gained from experimental studies, the therapeutic role of MSCs in liver regeneration must be further investigated, as the clinical evidence is still limited. As far as I know, to date, no clinical application has been performed.

#### **Adipose tissue-derived mesenchymal stem cells**

Adipose tissue is a source of MSCs that can be easily isolated, selected and induced into mature, transplantable hepatocytes. The fact that they are easy to procure *ex vivo* in large numbers makes them an attractive tool for clinical studies in the context of establishing an alternative therapy for liver dysfunction<sup>[153]</sup>. Adipose tissue-derived MSCs have immunomodulation, differentiation (plasticity), homing, revascularization, anti-apoptotic and tissue regenerating abilities<sup>[136]</sup>.

**Experimental results:** Transplanted adipose-derived MSCs through tail vein injection were able to differentiate into hepatocytes in BALB/c nude mice with CCl<sub>4</sub>-induced liver injury, and were able to function like human mature hepatocytes. Adipose-derived MSCs could be differentiated into hepatocytes within 13 d<sup>[154]</sup>. When approximately 10<sup>5</sup> of adipose-derived human MSCs (0.2 mL of the cell suspension *via* tail vein) transplanted by injection into mice with liver failure, the ammonia concentration fell to near normal levels within 24 h<sup>[153]</sup>. Of the various transplantation routes (tail vein, portal vein, and direct liver parenchymal injections), transplantation *via* the tail vein was found to be the most effective in reducing biochemical parameters in CCl<sub>4</sub>-induced liver failure in mice<sup>[155]</sup>.

**Clinical results:** In the study of Zhang *et al*<sup>[156]</sup> 30 chronic hepatitis B patients with decompensated livers received umbilical cord-derived MSC transfusion. No significant side effects or complications were observed. Liver function improved and the volume of ascites significantly decreased. Umbilical cord-derived MSC have also been found to be safe and beneficial in the treatment of the patients with acute-on chronic liver failure associated with hepatitis B virus infection. The cell transfusions significantly increased the survival rates in ACLF patients<sup>[157]</sup>.

#### **Bone marrow-derived hematopoietic stem cells**

In 1999 Petersen *et al*<sup>[42]</sup> and in 2000 Lagesaa *et al*<sup>[43]</sup> described the contribution of bone marrow-derived stem cells (BMSs) to liver regeneration. Data in the literature increasingly suggest bone marrow as a transplantable source of hepatic progenitors<sup>[158,159]</sup>. Initial reports of the hepatic potential of HSCs were later shown to have resulted from fusion between transplanted donor cells and resident recipient hepatocytes<sup>[48,160]</sup>. The authors analyzed sex-mismatched bone marrow and liver transplantations in rats<sup>[42]</sup>, mice<sup>[158]</sup> and humans<sup>[161]</sup>, and were able to show Y-chromosome-positive hepatocytes as single cells or small clusters in the recipients. Adjusted Y-positive hepatocyte and cholangiocyte engraftment ranged from 4% to 43%, and from 4% to 38%, respectively<sup>[160]</sup>.

**Experimental results:** Cantz *et al*<sup>[162]</sup> have investigated the contribution of intrasplenic bone marrow transplants or *in vivo* mobilized HSCs to the formation of hepatocytes in normal and injured liver by CCl<sub>4</sub>. They concluded that there is little or no contribution of BMSs to the regeneration of normal and injured livers in the animal models used. Kanazawa *et al*<sup>[163]</sup> also demonstrated that there is little or no contribution of BMCs to the replacement of injured livers (both acute and chronic) in three different models, as follows: CCl<sub>4</sub> treatment, albuminurokinase transgenic mouse, hepatitis B transgenic mouse. By contrast, Jang *et al*<sup>[164]</sup> reported that transplantation of a population of bone marrow purified stem cells promoted functional improvement in mice with CCl<sub>4</sub>-induced acute liver injury. Moreover, liver function was restored 2-7 d after transplantation. Fibrosis reduction was also reported in rats with CCl<sub>4</sub>-induced acute liver injury after bone marrow mononuclear cells transplantation *via* the portal vein. The general condition of the rats in the treatment group also improved markedly<sup>[165]</sup>. In the study of Shizhu *et al*<sup>[166]</sup> transplanted bone marrow mononuclear cells *via* tail veins of mice were found to populate the damaged liver around the portal and centrolobular regions, and they appeared to differentiate into albumin-producing hepatocyte-like cells. Animals that received bone marrow mononuclear cells also showed a trend toward improved liver enzymes, as well enhanced survival rates, relative to controls.

**Clinical results:** Although the results of experiments on rodents are conflicting, several clinical trials found that BMSCs were beneficial in the treatment of the patients with liver failure. Autologous BMSCs transplantation *via* the portal vein, peripheral vein or hepatic artery into patients with cirrhosis, resulted in improvement of liver function tests<sup>[167-171]</sup>. Clinical studies by Lyra *et al*<sup>[172,173]</sup> suggested the safety of autologous bone marrow-derived cells through a hepatic artery for chronic liver disease patients. In nine patients with alcohol-related cirrhosis, the reinfusion of CD34+ HSCs into the hepatic artery was well tolerated and beneficial to liver function<sup>[174]</sup>. However, in the study of Cauto *et al*<sup>[171]</sup>, one case of dissection of the hepatic artery and one case of Takotsubo syndrome occurred as early complications. A patient developed a cutaneous immunological disorder and another patient developed hepatocellular carcinoma 12 mo after infusion *via* the hepatic artery. A phase 1 trial using BMSCs injected *via* the hepatic artery after portal embolization was prematurely terminated when a patient with decompensated cirrhosis died from radio contrast nephropathy and hepatorenal syndrome<sup>[175]</sup>.

A recent case report described the use of autologous unsorted BMSCs as rescue treatment for hepatic failure in a 67-year-old man ineligible for liver transplantation<sup>[176]</sup>. Apparent rapid improvement in hepatic synthetic function was obtained after the portal venous infusion of the cells. A liver biopsy performed 20 d after cell transplant

was reported to show increased hepatocyte replication around necrotic foci. Salama *et al*<sup>[177]</sup> reported that near normalization of liver enzymes was observed in 54% of 90 patients with end-stage liver disease received GSF for five days followed by autologous CD34+ and CD133+ stem cell infusion in the portal vein. Similarly, in a phase I clinical trial of five patients with acute on chronic liver failure, administering G-CSF and then reinfusing the CD34+ cells improved liver function in more than 50% of cases during a 60-d follow-up<sup>[167]</sup>. The patients receiving autologous infusion of mobilized adult bone marrow derived CD34+ cells without G-CSF were monitored for up to 18 mo, which confirmed the safety of the procedure, with beneficial effects lasting around 12 mo<sup>[170]</sup>. Terai *et al*<sup>[169]</sup> implemented a clinical trial on nine patients with decompensated liver cirrhosis. These patients were infused with  $5.2 \pm 0.63 \times 10^9$  autologous bone marrow cells from the peripheral vein. At 24 wk after transplantation, significant improvements were observed.

#### Peripheral and umbilical blood stem cells

Stem cells derived from cord blood of human origin exhibit higher plasticity than the respective mouse or rat cells<sup>[178]</sup>. Like the BMSCs, cell fusion has been implicated as the mechanism by which human cells are seen in the recipient's liver. Some researchers observed cell fusion in most cells<sup>[179]</sup> and some claim no evidence of cell fusion<sup>[180]</sup>. Newsome *et al*<sup>[180]</sup> demonstrated that human umbilical cord-blood (hUCB)-derived cells could differentiate into hepatocytes after transplantation into immunodeficient mice. The percentage of human, compared with mouse, hepatocytes reached an average of 0.011% after 16 wk. Kögler *et al*<sup>[181]</sup> reported that these somatic multipotent stem cells could differentiate into hepatocytes after transplantation into a pre-immune fetal sheep model. Human hepatocytes constituted as much as 20% of the liver 11 mo after transplantation<sup>[182]</sup>.

**Experimental results:** Intraperitoneal administration resulted in a rapid liver engraftment using a model of hepatic damage induced by allyl alcohol in nonobese diabetic-severe combined immunodeficient (NOD/SCID) mice<sup>[178]</sup>. Hepatocyte-like cells, known as NeoHeps, which are derived from terminally differentiated peripheral blood monocytes, also seem to be very effective in treating experimental ALF in Wistar rats<sup>[183]</sup>.

**Clinical results:** In a clinical trial, 40 patients with HBV-related cirrhosis were randomized to receive G-CSF alone or in combination with the reinfusion of peripheral blood monocytes in the hepatic artery. Over a 6-mo follow-up, significant biochemical and clinical improvement was seen in both groups<sup>[184]</sup>. In a different setting, Gasbarini *et al*<sup>[176]</sup> transplanted peripheral blood stem cells into a single patient with ALF and showed improvement of liver function over 30 d, although the patient eventually succumbed to sepsis.

## CONCLUSION

Although several cell transplantation trials concerning different types of mature or progenitor/stem cells in rodents succeeded in improving liver failure, cell transplantation therapies for human liver disorders are still in the early stages of development. Animal models of small animals may not reproduce the clinical syndrome of LF adequately, and trials in large animal models are required. Also mechanisms concerning transplanted cell engraftment and proliferation in LF need further analysis. Most of these clinical trials have limitations, being performed on small groups of patients, with no controls and using outcome parameters that are easily biased. The current inability to track transplanted or infused cells in human subjects represents a major challenge in further developing and understanding stem cell therapies. Clinical trials should be planned, with the development of standardized protocols for standardized procedures to define the nature of cells, the patients enrolled, the transplantation procedure and pre-treatment of the liver, as well as standard data collection regarding efficacy, and possible side effects. The results of the experiments are promising; therefore, cell transplantation therapies should be the first choice in the treatment of acute or end-stage liver failure in the near future.

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## Risk of ileal pouch neoplasms in patients with familial adenomatous polyposis

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### Abstract

Restorative proctocolectomy is the most common surgical option for patients with familial adenomatous polyposis (FAP). However, adenomas may develop in the ileal pouch mucosa over time, and even carcinoma in the pouch has been reported. We therefore reviewed the prevalence, nature, and treatment of adenomas and carcinoma that develop after proctocolectomy in the ileal pouch mucosa in patients with FAP. In 25 reports that were reviewed, the incidence of adenomas in the ileal pouch varied from 6.7% to 73.9%. Several potential factors that favor the development of pouch polyps have been investigated, but many remain controversial. Nevertheless, it seems certain that the age of the pouch is important. The risk appears to be 7% to 16% after 5 years, 35% to 42% after 10 years, and 75% after 15 years. On the other hand, only 21 cases of ileal pouch carcinoma have been recorded in the literature to date. The diagnosis of pouch carcinoma was

made between 3 to 20 years (median, 10 years) after pouch construction. Although the risk of malignant transformation in ileal pouches is probably low, it is not negligible, and the long-term risk cannot presently be well quantified. Regular endoscopic surveillance, especially using chromoendoscopy, is recommended.

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**Key words:** Familial adenomatous polyposis; Restorative proctocolectomy; Ileal pouch; Ileal pouch-anal anastomosis; Ileo-rectal anastomosis; Adenoma; Adenocarcinoma; Pouch polyp; Pouch neoplasm

**Core tip:** To eliminate the risk of colorectal cancer, the majority of patients with familial adenomatous polyposis (FAP) are treated with restorative proctocolectomy and an ileal pouch-anal anastomosis. However, as these patients are followed-up for longer intervals, it has gradually become recognized that adenomas and adenocarcinomas may develop in the ileal pouch. If the standard-of-care surgery for FAP patients does not eliminate all cancer risk, surgical and follow-up strategies may need to be altered. In this review, we summarize the data from the published English literature regarding the incidence of adenomas and carcinomas in the ileal pouch after proctocolectomy in FAP patients.

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### INTRODUCTION

Familial adenomatous polyposis (FAP) is an inherited, autosomal-dominant disease caused by a germline muta-

tion of the adenomatous polyposis coli gene (*APC*)<sup>[1]</sup>. The phenotype is characterized by the development of hundreds of colorectal adenomas, leading to a 100% lifetime risk of colorectal cancer<sup>[2]</sup>. For this reason, a prophylactic colectomy is recommended for patients with FAP to prevent the development of colorectal cancer. The main surgical strategy in patients with FAP is restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA)<sup>[3-6]</sup>. As originally described by Parks *et al*<sup>[7]</sup>, IPAA included an anal mucosectomy to eliminate the risk of malignancy in the remaining anorectal mucosa. However, many surgeons now prefer to preserve the anal transition zone (ATZ) during the double-stapled IPAA technique because of its simplicity and better functional outcome<sup>[8-10]</sup>. The trade-off is a risk of neoplasia developing in the retained ATZ mucosa<sup>[8]</sup>, with a 10%-15% incidence of adenoma<sup>[8,11-13]</sup>. Another widely accepted surgical procedure is colectomy with ileorectal anastomosis (IRA), performed when there are few polyps in the rectum. The major advantage of IRA is preservation of the rectal innervation, with subsequent better quality of life. However, continuing endoscopic surveillance for adenomas in the rectum is necessary, and there is a 13%-25% cumulative risk of rectal cancer after 15-25 years despite surveillance<sup>[14-16]</sup>. On the other hand, it has been thought that IPAA theoretically eliminates the risk of colorectal cancer and adenomas, and perhaps the need for further lower gastrointestinal surveillance. However, there are recent reports of adenomas or carcinomas developing not only in the residual rectal mucosa or anastomosis after IRA, but also in the ileal pouch mucosa after IPAA<sup>[17-39]</sup>. In addition, there are several reports of cancers arising from the ileal pouch mucosa, as opposed to the anastomotic site, in patients with FAP<sup>[31,36,37,40-47]</sup>.

The aim of this review is to describe the prevalence, nature, and treatment of adenomas and carcinoma developing in the ileal pouch mucosa after proctocolectomy in patients with FAP.

## INCIDENCE OF ILEAL POUCH ADENOMAS

The basic premise underlying the popularity of IPAA in patients with FAP is that it results in a significantly lower risk of rectal cancer than IRA. Although this is likely to be true, the risk of pouch polyposis and pouch cancer has not been recognized so far. After the advent of pouch surgery, several reports of adenomatous polyps in the pouches of FAP patients have appeared in the literature. In 1982, Beart *et al*<sup>[17]</sup> first described a FAP patient with continent ileostomy, in whom a large sessile tubulovillous adenoma and multiple smaller adenomatous polyps developed. Since then, there have been at least 25 reports of ileal pouch adenomas developing in these patients (Table 1). We reviewed only those studies that clearly described adenomas appearing within the ileal pouch mucosa. These included 8 case reports, 7 retrospective studies, and 10 prospective studies. The incidence of adenomas in the ileal pouch varied from 6.7%

to 73.9%. This variation in the prevalence of ileal pouch adenomatosis could be due to differences in the way endoscopy was performed in the various studies. Adequacy of bowel preparation, type of instrument employed, use of chromoendoscopy, and other miscellaneous factors influence the detection rates of pouch polyps. Good bowel preparation and a flexible video colonoscope are essential in identifying pouch polyps because most ileal adenomas are flat or sessile, measuring only 1 mm to 3 mm in diameter, and having a morphology different from large bowel adenomas<sup>[36]</sup>. Therefore, a careful examination of the entire pouch, anal canal, and pouch-anal anastomosis should be performed. Although Polese *et al*<sup>[25]</sup> reported the prevalence of ileal pouch adenomas as 6.7%, these investigators used a rigid sigmoidoscope with enema preparation, without chromoendoscopy. These technical factors may explain the low prevalence of ileal pouch adenomas in the study by Polese *et al*.

The only meaningful estimates of adenoma incidence have come from prospective studies, with all 10 prospective studies showing that the incidence of pouch adenomas increases with the follow-up duration<sup>[4,21,22,24,28,29,31,32,35,36]</sup>. The risk appears to be 7% to 16% after 5 years, 35% to 42% after 10 years, and 75% after 15 years. The great majority of pouch polyps described in the literature are small, tubular adenomas with mild atypia. The incidence of adenoma with advanced pathology (size > 1 cm, villous pattern, or moderate-to-severe dysplasia) is less. According to Banasiewicz *et al*<sup>[35]</sup>, the estimated frequency of low-grade dysplasia 4 years after IPAA surgery is 4%, and increases to 50% after 15 years. The same percentage of patients, but with high-grade dysplasia, develop high-grade dysplasia 2.5 years later, that is to say, 17.5 years after IPAA, and with neoplasia 18.5 years after the above procedure. The full impact of pouch polyposis will not be fully understood until most pouch patients reach a follow-up duration of 20 to 40 years<sup>[48]</sup>.

## RISK FACTORS FOR DEVELOPMENT OF ADENOMAS IN THE ILEAL POUCH

### Phenotype

At present it does not seem possible to predict who is at risk for developing polyps in the pouch. Some studies show that there is no apparent high-risk phenotype for the development of ileal polyps<sup>[22,24,30,33]</sup>. However, other studies describe risk factors that favor the development of pouch polyposis. Parc *et al*<sup>[49]</sup> observed that patients with pouch polyps are younger, and have a longer follow-up period since IPAA, than patients without pouch polyps. A more aggressive disease requiring earlier surgery could explain these features. However, Groves *et al*<sup>[28]</sup> analyzed the risk factors by using logistic regression, and found that age was a more significant predictor of pouch adenomas than the follow-up period, sex, or type of primary or secondary procedures. Groves *et al*<sup>[28]</sup> reported that, in their experience, all patients older than 60 years

**Table 1 Summary of 25 reports of ileal pouch adenomas in familial adenomatous polyposis**

Ref.	Study	Year	Operation	n	Type of pouch	Findings	% of adenoma	Time to neoplasia
Beart <i>et al</i> <sup>[17]</sup>	Case	1982	Kock	1	Kock pouch	Tubulovillous Adenomas		6 yr
Wolfstein <i>et al</i> <sup>[18]</sup>	Case	1982	IAA	2	Soave	Adenomas in 2 pts		3 and 7 yr
Shepherd <i>et al</i> <sup>[19]</sup>	Retro	1987	IPAA	12	N	Tubular Adenoma in 2 pts	16.7	
Stryker <i>et al</i> <sup>[20]</sup>	Case	1987	Brooke ileostomy	1	Kock pouch	Tubular Adenomas		12 yr
Nugent <i>et al</i> <sup>[16]</sup>	Retro	1993	IPAA	38	N	Tubular Adenoma in 5 pts	13.2	4 yr (1-7)
Bertoni <sup>[21]</sup>	Pros	1995	IAA	3	N	Tubular Adenoma in 2 pts	66.7	53.7-75.4 mo
Wu <i>et al</i> <sup>[22]</sup>	Pros	1998	IPAA	26	S/J-pouch 9/16, 1 N	Tubular Adenoma in 11 pts	42.3	1-14 yr
Valle <i>et al</i> <sup>[23]</sup>	Case	2001	IPAA	5	N	Adenomas	20	5 yr
Thompson-Fawcett <i>et al</i> <sup>[24]</sup>	Pros	2001	Kock 5, IPAA 28	33	N	Adenoma in 14 pts (with microadenoma 20 pts)	42.4 (60.6)	7 yr (1-19)
Parc <i>et al</i> <sup>[4]</sup>	Pros	2001	IPAA	85	J-pouch	30 pts (28 grossly visible, 2 microadenoma)	35.3	Cumulative risk of 7%, 35%, and 75% at 5, 10, and 15 yr
Polese <i>et al</i> <sup>[25]</sup>	Retro	2002	IPAA	46	S/W/J-pouch 2/1/43, Kock 4	Adenoma in 2 of 30 pts	6.7	risk of Adenoma after 8 yr; 20%, (9-11 yr)
Beveridge <i>et al</i> <sup>[26]</sup>	Case	2004	IPAA	2	J-pouch 1, Kock 1	2 large VA, VA		4-10 yr
Vrouenraets <i>et al</i> <sup>[27]</sup>	Case	2004	IPAA	1	J-pouch	Adenomas with focal severe dysplastic change		6 yr
Groves <i>et al</i> <sup>[28]</sup>	Pros	2005	Kock 4, IPAA 56	60	W/J-pouch 13/43, Kock 4	Mild dysplasia 23, more advanced 11	56.7	6 yr (1-17)
Nilubol <i>et al</i> <sup>[29]</sup>	Pros	2007	IPAA	10	N	Tubular Adenoma in 1 of 9 pts	11.1	11.3 yr
Moussata <i>et al</i> <sup>[30]</sup>	Retro	2008	IPAA	23	N	Low-grade 16, high-grade 1	73.9	4.76 yr (1-14)
Friederich <i>et al</i> <sup>[31]</sup>	Pros	2008	IPAA	212	N	Adenoma 74 pts, AAP 25 pts	46.7	Cumulative risk of 16% and 42.2% at 5 and 10 yr
Schulz <i>et al</i> <sup>[32]</sup>	Pros	2008	IPAA	35	N	Low-grade 8	22.8	mean of 5 yr
Tajika <i>et al</i> <sup>[33]</sup>	Retro	2009	Kock 8, IPAA 16	24	J-pouch	16 pts, (advanced 1, carcinoma 2)	66.7	Cumulative risk of 13%, 43%, and 72% at 5, 10, and 20 yr
Kang <i>et al</i> <sup>[34]</sup>	Case	2010	Kock	2	Kock pouch	2 (the largest one is 15mm in size)		
Banasiewicz <i>et al</i> <sup>[35]</sup>	Pros	2011	IPAA	165	J-pouch	Low-grade 13, high-grade 8, neoplasia 5	15.8	Cumulative risk of 50%, Low-grade at 15 yr, high-grade at 17.5 yr, neoplasia 18.5 yr
Tonelli <i>et al</i> <sup>[36]</sup>	Pros	2012	IPAA	69	S/J-pouch 25/29, SIMM 15	Adenoma 25 pts, carcinoma 2 pts	39.1	Cumulative risk of 28.5% at 5 yr
Makni <i>et al</i> <sup>[37]</sup>	Case	2012	IPAA	1	J-pouch	Adenoma and carcinoma		10 yr
Wasmuth <i>et al</i> <sup>[38]</sup>	Retro	2013	IPAA	61	N	14 pts	23	Estimated cumulative rate of first Adenoma diagnosed was 38%
Pommaret <sup>[39]</sup>	Retro	2013	IPAA	118	J-pouch	57 pts (12 advanced Adenomas)	48.3	15 yr

Study: Type of study; Retro: Retrospective series; Pros: Prospective series; n: Number of patients in the study; IPAA: Ileal pouch-anal anastomosis; Kock: Kock continent ileostomy; N: Not described; AV: Anal verge; pts: Patients.

will develop polyps in the pouch. Tonelli *et al*<sup>[36]</sup> reported that an age of more than 50 years was associated with pouch adenomas, but not sex or elapsed time since restorative proctocolectomy.

Several studies found that the severity of duodenal polyposis was related to the presence of pouch adenomatous polyps. In a multivariate analysis of 118 FAP patients who had undergone surgery, Pommaret *et al*<sup>[39]</sup> discovered that the presence of advanced duodenal adenomas was an independent risk factor for the development of pouch adenomas, in addition to follow-up duration. Tonelli *et al*<sup>[36]</sup> found that patients affected by pouch adenomas had high polyp counts (> 1000) at colectomy, as well as duodenal adenomas.

Several potential factors that favor the development of pouch polyposis have been investigated; a number of them remain controversial, although it seems certain that

the age of the pouch is important.

### Pathogenesis

The mucosa of the ileal pouch may be subjected to not only the tumorigenic consequences of APC gene mutations<sup>[50]</sup>, but also to luminal factors due to fecal stasis, which may also exert an important effect. Fecal stasis, such as occurs in a reconstructed pouch, may promote neoplastic changes in the ileal mucosa. Several authors have implicated colonic metaplasia of the ileal mucosa as a precursor for the development of ileal adenomas<sup>[19,51,52]</sup>, and even carcinomas in the surgically constructed pouches of patients with FAP<sup>[53-55]</sup>. Colonic metaplasia was frequently recognized even in earlier descriptions of the changes observed in the ileal pouch mucosa. Some authors have considered colonic metaplasia as an adaptive response of the ileal pouch to its role as a neo-

rectum<sup>[18,19,56,57]</sup>. Further investigations have shown that colonic transformation is only partial. Small-bowel brush border disaccharidase activity is preserved, as is the ability to absorb vitamin B12, D-xylose, phenylalanine, and bile acids<sup>[52,58-60]</sup>. The mucosal changes described as colonic metaplasia are likely a response to chronic inflammation caused by changes in the luminal contents due to stasis. In FAP, these changes may, at least in theory, favor the development of adenomas in a region of the gut where they are not usually observed. There is an increase in the concentration of luminal short chain fatty acids to levels that are seen in the colon<sup>[61]</sup>, an increase in anaerobic bacterial counts with a more colonic type flora<sup>[62,63]</sup>, and increased deconjugation and dehydroxylation of bile acids by the anaerobic bacteria<sup>[64]</sup>. In particular, deoxycholic acid and lithocholic acid, which are known carcinogens, have concentrations several times higher in an ileal pouch than in an end ileostomy<sup>[65]</sup>. On the other hand, reduction of glutathione S-transferase (GST) detoxification activity in the pouch compared with the afferent ileal loop after IPAA may promote tumorigenesis<sup>[64]</sup>.

### APC gene mutations

FAP develops due to a dominant autosomal mutation of the *APC* gene in more than 80% of patients<sup>[65]</sup>. Recently, it has been discovered that a biallelic mutation of the *MUTYH* gene might exist in 5% of patients with colorectal polyposis and 20% of FAP patients, with no *APC* mutation found<sup>[66]</sup>. Many researchers have investigated *APC* gene mutations in pouch patients with FAP, although none has demonstrated obvious genotype-phenotype correlations that would predict the development of pouch adenomas<sup>[28,30,36,49]</sup>. Hence, the available evidence suggests that systematic surveillance of all patients who undergo IPAA is necessary. Targeted surveillance of a defined subgroup of patients is currently not feasible.

### Type of pouch and anastomosis

There are different pouch configurations. Parks *et al.*<sup>[7]</sup> originally devised a triple-limb S-shaped pouch. This pouch was relatively complicated to construct, and suffered from kinking of the efferent limb if it was left too long<sup>[67]</sup>. Alternative designs have included the high-capacity W-pouch, the H-pouch and the J-pouch. The majority of surgeons now favor the J-pouch due to ease of construction, economical use of the terminal ileum, and reliable emptying<sup>[68]</sup>. Functional results are equal to those of other reservoir designs<sup>[69-71]</sup>. The pouch is formed from the terminal 40 cm of ileum, using several applications of a linear-cutting stapler to join the anti-mesenteric borders of two 20-cm ileal limbs.

Several authors have shown that there is no apparent relationship between the development of pouch polyps and the type of ileal pouch construction<sup>[36]</sup> or the suture used (hand-sewn or stapled)<sup>[25,28,36]</sup>. However, other authors have reported that patients with a stapled IPAA are at a significantly increased risk of developing adenomas at the anastomotic site: 1.5% to 20.9% *vs*

27% to 66% for hand-sewn and stapled anastomoses, respectively<sup>[9,11,22,36,72,73]</sup>. These data suggest that a hand-sewn IPAA may be a preferable strategy for decreasing the occurrence of adenomas at the anastomotic ileo-anal site. Recently, Wasmuth *et al.*<sup>[38]</sup> evaluated the differences between adenoma formation at the anastomotic site and in the ileal pouch after IPAA, with or without mucosectomy. These investigators found that an occurrence of adenomas at the anastomotic site was significantly reduced after mucosectomy. However, there was no difference in the occurrence of ileal pouch adenomas between patients who underwent mucosectomy and those who retained a rectal mucosal remnant (8/39 *vs* 6/22;  $P = 0.57$ )<sup>[38]</sup>.

### Pouchitis

In patients with ulcerative colitis, concern about the risk of neoplasia in ileal pouches was raised after observing a combination of histologic changes in the ileal mucosa of the pouch, including villous atrophy, inflammation and metaplasia<sup>[74-76]</sup>. These transformations in the ileal pouches are likely caused by the chronic inflammatory state. The inflammatory process in pouchitis may lead to dysplasia<sup>[47]</sup> and loss of heterozygosity, consistent with precancerous lesions of the colon<sup>[77]</sup>. Thus, a dysplasia-to-neoplasia progression can occur in ileal pouches and can lead to cancer of the pouch. The cumulative risk of pouchitis is up to 50% in patients with ulcerative colitis<sup>[78-81]</sup>, with most patients experiencing at least one episode of pouchitis during the first ten years after surgical pouch construction. In contrast, the pouchitis rate is below 25% in patients with FAP<sup>[4-6,29,33,35]</sup>. Banasiewicz *et al.*<sup>[35]</sup> analyzed the frequency and progression of dysplasia and inflammation in the intestinal pouch of FAP patients after restorative proctocolectomy. Although these authors diagnosed pouchitis in 20.6% of patients after restorative proctocolectomy, no relationship was found between pouchitis and pouch dysplasia in FAP patients.

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## THE PREVALENCE OF ADENOMAS IN THE PRE-POUCH ILEUM

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Although it is currently recognized that adenomas may develop in the ileal pouch, the risk of adenomas occurring in the afferent ileal loop above the pouch is unclear. The incidence of adenomas above the IPAA pouch was rarely recognized previously, and it seemed to be low, with reported figures ranging from 4% to 16%<sup>[22,28,33,82]</sup>. The majority of pre-pouch ileal adenomas have measured 4 mm or smaller. Pommaret *et al.*<sup>[39]</sup> reported that only nine (6.5%) of 118 patients had afferent ileal loop adenomas after an IPAA. The only independent predictive factor for the occurrence of afferent ileal loop adenoma was found to be the presence of pouch adenomas (OR: 2.16; 95%CI: 0.17-26.98;  $P = 0.007$ ). Pommaret *et al.*<sup>[39]</sup> concluded that because afferent ileum loop adenomas are rare and have an unclear pathologic significance, there is no justification for their systematic search, particularly among patients without any duodenal or pouch adeno-

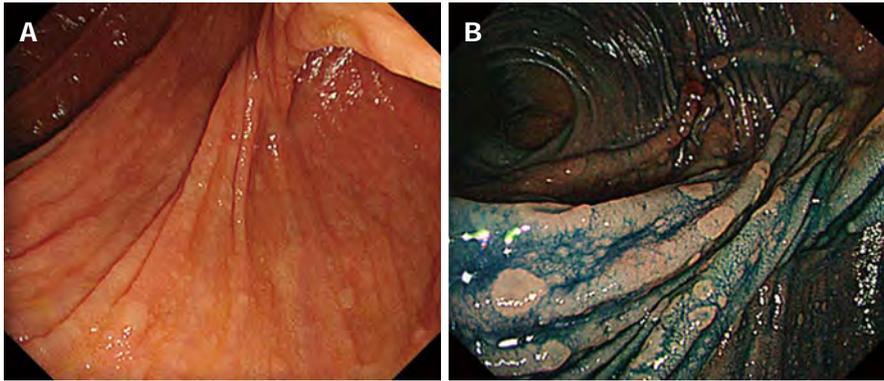


Figure 1 Endoscopic view of ileal pouch adenomas in patients with familial adenomatous polyposis. A: Multiple white flat lesions are observed in the ileal pouch mucosa; B: Multiple sessile polyps are revealed by indigo carmine. The data available from reference [33].

mas. In cases of extensive pouch polyposis with a significant cancer risk, this viewpoint could allow clinicians to consider resection of the adenomatous pouch, with construction of a new one using the afferent ileum.

## SURVEILLANCE OF THE ILEAL POUCH

Saurin *et al.*<sup>[83]</sup> described methods of surveillance and therapeutic indications in FAP patients following colectomy. Although there are no validated data in the literature, on the basis of expert opinion, endoscopic surveillance is performed at 6 mo, 1 year, and then every 2 years after surgery. In the presence of polyps with high-grade dysplasia, and/or polyps > 1 cm size, and/or presence of large polyp number (> 30), surveillance should be repeated every 6 mo<sup>[83]</sup>. Many authors have performed pouch endoscopy every 6-12 mo after surgery<sup>[30,33,35,36,38,84]</sup>. Optimum bowel preparation and the use of indigo carmine surface staining are necessary<sup>[31-33,35]</sup>. The main utility of chromoendoscopy is to highlight the small lymphoid lesions that are characteristic of the terminal ileum in FAP patients, and to distinguish flat polyps (Figure 1). In the experience of the Dutch Registry<sup>[31]</sup>, indigo carmine chromoendoscopy significantly increases the detection rates of adenomas < 5 mm in size. Investigators with the Dutch Registry found that 75.7% of FAP patients harbored adenomas in the pouch at a median follow-up duration of 8 years after IPAA<sup>[31]</sup>. Because some patients have a stricture at the anal anastomosis, a pediatric colonoscope or gastroscopie may sometimes be required<sup>[35]</sup>.

## TREATMENT OF ADENOMAS IN THE ILEAL POUCH

According to Saurin *et al.*<sup>[83]</sup>, no systematic endoscopic treatment of adenomas of the ileal pouch or afferent loop can be recommended. For large adenomas (> 1 cm), or in patients with high-grade dysplasia, endoscopic resection must be considered; however, a skilled team is needed because of the thin ileal mucosa<sup>[83]</sup>. Our current strategy in patients with IPAA is regular follow-up starting at 1 year after surgery, and then every year

thereafter<sup>[33]</sup>. If adenomas are observed in the pouch, we recommend endoscopic resection or argon plasma coagulation where feasible, and then follow-up every 6 mo. Other reports<sup>[30,35,36,84]</sup> also describe polypectomy of large polyps, and ablation by fulguration or electrocoagulation for small lesions. In patients having extensive pouch polyposis with no possibility of endoscopic treatment, together with invasive cancer, pouch excision and terminal ileostomy has to be considered<sup>[40,83]</sup>.

Although there have been some reports suggesting the efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) in suppressing the development of ileal pouch adenomas<sup>[32,48,85]</sup>, this has not been systematically studied<sup>[34]</sup>.

## THE PREVALENCE OF ADENOCARCINOMAS IN THE ILEAL POUCH

The progression from a dysplastic lesion in the ileal pouch to invasive carcinoma appears to be rare, occurring in no more than 1% of patients with ileal pouches<sup>[31]</sup>. However, in the past few years, several cases of carcinoma have been observed after IPAA, although the majority of them occurred at the level of the anal canal<sup>[8,9,13,27,38,86-91]</sup>. Patients with either hand-sewn or stapled IPAA are at risk for developing a carcinoma in any residual rectal mucosa that harbors dysplasia or is prone to dysplasia. To date, only 21 cases of ileal pouch carcinoma have been recorded in the literature (Table 2). No relationship has been found between the occurrence of pouch carcinoma and the shape (J or S) of the pouch, or the type (hand-sewn or stapled) of IPAA<sup>[36]</sup>. These pouch cancers have clearly appeared in the ileal pouch, and not in the ATZ. The time elapsed between pouch construction and diagnosis of pouch carcinoma has been between 3 and 20 years (median, 10 years).

It is noteworthy that in at least seven patients, the development of advanced cancer was detected within a very short interval-within 1 year since last pouch endoscopy<sup>[25,36]</sup>. Furthermore, in four of the patients, ileal polyps

Table 2 Summary of 21 cases of ileal pouch cancer in familial adenomatous polyposis

Ref.	Year	Sex	Operation	Type of pouch	Staging of initial surgery	Age of pouch (yr)	Shape	Size (mm)	Staging of pouch cancer	No. of pouch polyps	Time to cancer (yr)	Outcome	Interval since last endoscopy (yr)
Bassuini <i>et al</i> <sup>[440]</sup>	1996	M	IPAA	/handsewn	No cancer	28	Large polypoid	N	T3,N+	N	3	N	No follow-up
Palkar <i>et al</i> <sup>[441]</sup>	1997	F	IPAA	J-pouch handsewn	No cancer	39	Large polypoid	40 × 35	T4N0	Exist	4.7	Alive	0.3
Kim <i>et al</i> <sup>[442]</sup>	1997	N	N	N	N	N	N	N	N	N	N	N	N
Cherki <i>et al</i> <sup>[443]</sup>	2003	F	IPAA	J-pouch handsewn	TisN0M0	35	N	N	T3N1M1	N	3.5	Died	0.5
Linehan <i>et al</i> <sup>[444]</sup>	2007	M	IPAA	/double stapled	Dukes A	30	N	N	T3N0	N	9	Alive	No follow-up
Friederich <i>et al</i> <sup>[311]</sup>	2008	M	IPAA	/handsewn	No cancer	21.3	N	N	Dukes C	0	14	N	4.4
		M	IPAA	/stapled	No cancer	26.7	N	N	Dukes B	0	10	N	2.1
		M	IPAA	/handsewn	No cancer	16	N	N	Dukes B	N	16	N	No follow-up
		F	IPAA	/stapled	No cancer	29.6	N	N	Dukes B	exist	6	N	0.6
Tajika <i>et al</i> <sup>[45]</sup>	2009	F	IPAA	J-pouch/handsewn	TisN0M0	46	Type 2	30 × 25	T4N2M0	0	8.6	Died 3Y	0.75
		M	Kock	Kock/handsewn	No cancer	48	Type 1	40 × 35	T3N0M0	10 <	20	Died by U	No follow-up
Ault <i>et al</i> <sup>[46]</sup>	2009	M	IPAA	S-pouch/handsewn	Four cancer	61	ND	20-30	T2N1	N	11	Died by U	6
		F	IPAA	ND	No cancer	40	Type 1	N	N	N	13	meta	No follow-up
Lee <i>et al</i> <sup>[47]</sup>	2009	F	IPAA	J-pouch/handsewn	T2N0	56	Type 2	30 × 25	T3N2	0	7	meta 2Y	4
Banasiewicz <i>et al</i> <sup>[35]</sup>	2011	N	IPAA	J-pouch/handsewn	N	N	N	N	N	N	N	N	N
		N	IPAA	J-pouch/handsewn	N	N	N	N	N	N	N	N	N
		N	IPAA	J-pouch/handsewn	N	N	N	N	N	N	N	N	N
		N	IPAA	J-pouch/handsewn	N	N	N	N	N	N	N	N	N
Tonelli <i>et al</i> <sup>[36]</sup>	2012	M	IPAA	S-pouch/handsewn	No cancer	26	Type 2	20 <	T3N0M0	ND	3	Died 6 mo	1
		F	IPAA	S-pouch/handsewn	TisN0M0	47	II a + II c	N	T2N0M0	0	11	Alive at 56 mo	0.5
Makni <i>et al</i> <sup>[37]</sup>	2012	F	IPAA	J-pouch/handsewn	No cancer	26	N	20	N	Many	10	Died 1Y+	0.66

M: Male; F: Female; IPAA: Ileal pouch-anal anastomosis; Kock: Kock continent ileostomy; N: Not described; AV: Anal verge; U: Unrelated disease; Time to cancer: Interval between cancer diagnosis and pouch construction.

were not found during endoscopic follow-up until the development of pouch carcinoma. It seems that neoplasia that appears in the ileal pouch may not always follow the classic adenoma-carcinoma sequence. We strongly recommend a strict surveillance program for FAP patients, including annual flexible colonoscopy, irrespective of the phenotype and genotype.

## IRA VS IPAA

In a review of 12 studies containing 1002 patients with FAP (53.4% IPAA, 46.6% IRA), Aziz *et al*<sup>[92]</sup> showed that bowel frequency, nocturnal defecation, and incontinence rates were significantly less in IRA patients, although fecal urgency was less among IPAA patients. There was no significant difference between IPAA and IRA in terms of sexual dysfunction, dietary restrictions, or postoperative complications. In their review, Aziz *et al*<sup>[92]</sup> could not identify any malignancies in IPAA patients, and rectal cancer was a diagnosis only in the IRA patients (5.5%). At the present time, IPAA anastomosis is recommended by a majority of surgical teams as the preferred option for FAP patients<sup>[4-6,10,92,93]</sup>. Although cancer formation after IPAA in patients with FAP may be rare, it is of concern that several of the recently reported patients had an ad-

vanced stage at diagnosis, with poor outcome.

Recent reports of a high frequency of adenomas after IPAA, together with favorable functional outcomes in patients who underwent IRA, may lead to reconsideration of the latter surgical option for some FAP patients, especially when quality of life and fertility criteria are taken into account<sup>[30]</sup>. In clinical practice, the results of preoperative evaluation of the rectal stump using indigo carmine chromoendoscopy are a major deciding factor. A limited number (< 10-20) of rectal polyps without any cancer would lead to preservation of the rectal stump in a majority of patients<sup>[94,95]</sup>. However, with the availability of better endoscopic instruments and resection techniques, and the possibility of enhanced post-operative surveillance, > 20 rectal polyps and/or non-invasive cancers can now also be managed endoscopically. So, there is a possibility that the criteria for IRA will expand in the near future. Of course, based on clinical and genetic data, a stepwise surgical strategy with a primary IRA followed at a later age by a secondary proctectomy and IPAA could be proposed<sup>[5]</sup>.

An ongoing multicenter study in Japan is being conducted by Ishikawa *et al*<sup>[96]</sup> under the title "Intervention trial for colorectal cancer prevention by endoscopic polypectomy in patients with familial adenomatous pol-

yposis" (UMIN000009365). The aim of this study is to evaluate the usefulness and safety of thorough endoscopic polypectomy in FAP patients who have (or had)  $\geq 100$  colonic adenomas and who refuse surgery, as well as post-operative patients who have (or had)  $\geq 100$  colonic adenomas and who have  $\geq 10$  cm of remnant colon.

## CONCLUSION

The development of adenomas with high-grade dysplasia and carcinoma in the ileal pouch is an important issue because the choice between IPAA and IRA is based mainly on the expected low risk of cancer development after the former surgery. Although the risk of malignant transformation in ileal pouches is probably low, it is not negligible, and the long-term risk cannot presently be well quantified. IPAA will not prevent cancer development in the terminal remnant intestine, and patients who undergo IPAA require regular follow-up similar to patients who receive IRA. A detailed analysis of the phenotypes and mutations in the *APC* gene of patients with FAP may allow tailoring of the surgical options in the future.

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## Clinical applications of next-generation sequencing in colorectal cancers

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### Abstract

Like other solid tumors, colorectal cancer (CRC) is a genomic disorder in which various types of genomic alterations, such as point mutations, genomic rearrangements, gene fusions, or chromosomal copy number alterations, can contribute to the initiation and progression of the disease. The advent of a new DNA sequencing technology known as next-generation sequencing (NGS) has revolutionized the speed and throughput of cataloguing such cancer-related genomic alterations. Now the challenge is how to exploit this advanced technology to better understand the underlying molecular mechanism of colorectal carcinogenesis and to identify clinically relevant genetic biomarkers for diagnosis and personalized therapeutics. In this review, we will introduce NGS-based cancer genomics studies focusing on those of CRC, including a recent large-scale

report from the Cancer Genome Atlas. We will mainly discuss how NGS-based exome-, whole genome- and methylome-sequencing have extended our understanding of colorectal carcinogenesis. We will also introduce the unique genomic features of CRC discovered by NGS technologies, such as the relationship with bacterial pathogens and the massive genomic rearrangements of chromothripsis. Finally, we will discuss the necessary steps prior to development of a clinical application of NGS-related findings for the advanced management of patients with CRC.

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**Key words:** Next-generation sequencing; Cancer genomics; Colorectal cancers; Personalized medicine; The cancer genome atlas

**Core tip:** Next-generation sequencing (NGS)-driven genomic analyses are facilitating the genomic dissection of various types of human cancers, including colorectal cancer (CRC). This review contains an up-to-date summary of recent NGS-based CRC studies and an overview of how these efforts have advanced our understanding of colorectal carcinogenesis with novel biomarkers for genome-based cancer diagnosis and personalized cancer therapeutics.

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### INTRODUCTION

Colorectal cancers (CRC) are the third most common human malignancy, and are also the leading cause of cancer-related deaths worldwide<sup>[1]</sup>. Early detection of premalignant

**Table 1** The next-generation sequencing platforms for cancer genome analysis

NGS types	Whole genome sequencing	Exome sequencing	Epigenome sequencing <sup>1</sup>	RNA-seq
Source	Genomic DNA	Genomic DNA (targeted)	Genomic DNA (targeted)	RNA
Alteration types	Point mutations and indels, rearrangements <sup>2</sup> , DNA copy number changes	Point mutations and indels	DNA methylation and posttranscriptional histone modifications	Gene fusions <sup>3</sup> , alternative splicing events, point mutations and indels

<sup>1</sup>Epigenome sequencing can be classified into chromatin immunoprecipitation (ChIP)-based methods to detect genomic domains with epigenetic modifications<sup>[80]</sup> and more direct DNA methylation sequencing such as bisulfite-sequencing<sup>[59,60]</sup>. <sup>2</sup>From whole genome sequencing data, the genomic rearrangements and DNA copy number changes are generally detected by paired-end mapping<sup>[81]</sup> and read-depth based methods<sup>[82]</sup>, respectively; <sup>3</sup>Gene fusions can also be identified by paired-end, high-coverage whole genome sequencing, but the transcription-related events such as exon skipping or other alternative splicing events can only be identified by RNA-seq. NGS: Next-generation sequencing.

**Table 2** The list of next-generation sequencing-based studies of colorectal cancer genomes

Ref.	NGS types	Major findings	Alteration types and software used
Bass <i>et al</i> <sup>[16]</sup>	WGS (9 pairs; tumor-matched normal)	Oncogenic fusion ( <i>VT11A-TCG7L2</i> )	Point mutations (MuTect <sup>[83]</sup> ) Indels (Indelocator) <sup>1</sup> Rearrangements (dRanger) <sup>1</sup>
TCGA consortium	WGS (97 pairs; low-pass) RNA-seq (218 tumors) Exome-seq (254 pairs)	See main text	Point mutations (MuTect) Recurrent mutations (MutSig) <sup>1</sup> DNA copy numbers (BIC-seq <sup>[82]</sup> ) Rearrangements (BreakDancer <sup>[81]</sup> )
Timmermann <i>et al</i> <sup>[84]</sup>	Exome-seq (2 pairs, one MSI-H and one MSS)	Comparison of mutation spectrum between MSI-H and MSS CRC genomes	Point mutation and indel (Vendor-provided GS reference mapper, Roche)
Zhou <i>et al</i> <sup>[85]</sup>	Exome-seq (1 series: normal-adenoma-adenocarcinoma)	Comparison of benign and malignant CRC genomes in the same patient	Point mutation and indel (Samtools <sup>[86]</sup> )
Kloosterman <i>et al</i> <sup>[73]</sup>	WGS (4 pairs; primary-metastasis-matched normals) Targeted 1300 genes (4 pairs)	Comparison of primary or metastatic CRC genomes	Chromothripsis and mutations (Burrow-Wheeler aligner <sup>[87]</sup> based in-house tools)
Brannon <i>et al</i> <sup>[88]</sup> (Proceedings)	Targeted 230 genes (50 pairs: primary-metastasis-matched normals)	Comparison of primary or metastatic CRC genomes	IMPACT (integrated mutation profiling of actionable cancer targets)
Yin <i>et al</i> <sup>[89]</sup>	RNA-seq (2 pairs)	RNA-seq based mutation study	Point mutations and indels (Samtools)

<sup>1</sup>Description of the software is available at <https://confluence.broadinstitute.org/display/CGATools/>. NGS: Next-generation sequencing; CRC: Colorectal cancer; TCGA: The cancer genome atlas; MSI: Microsatellite instability.

nant lesions such as adenomatous polyps has decreased the risk of CRCs<sup>[2]</sup>, however, cases which are initially undetected and progress to advanced CRC with distant metastasis are still unfortunately incurable<sup>[3]</sup>. The development of CRC is a complex and heterogeneous process arising from an interaction between multiple etiological factors, including genetic factors<sup>[4]</sup> and environmental factors such as diet and lifestyle<sup>[5]</sup>. Recently, significant progress has been made in the characterization of genetic and epigenetic alterations in CRC genomes in support of the genomic view of colorectal carcinogenesis. Like other types of human solid tumors, CRC genomes harbor various types of genomic alterations ranging from small-scale changes (*i.e.*, point mutations or small indels) to large-scale chromosomal copy number changes or rearrangements. Some of these alterations may contribute to colorectal carcinogenesis as oncogenic drivers, but the full spectrum of driver genomic alterations in CRC genomes is still incomplete.

For decades, the genome-wide profiling of cancer genomes has been mainly conducted using hybridization-based microarray technologies (*i.e.*, expression microarrays and array-based comparative genomic hybridization)<sup>[6,7]</sup> or low-throughput Sanger sequencing<sup>[8]</sup>. Recently,

the advancement of DNA sequencing technologies - next-generation sequencing (NGS) - has revolutionized the speed and throughput of DNA sequencing<sup>[9,10]</sup>. Table 1 lists the NGS platforms widely used in the characterization of cancer genomes. Since the first attempt at cancer genome sequencing using NGS technology<sup>[11]</sup>, successful sequencing by NGS has been accomplished in many major human cancer types<sup>[12,13]</sup> including gastrointestinal malignancies such as esophageal<sup>[14]</sup>, gastric<sup>[15]</sup>, colorectal<sup>[16]</sup>, and hepatocellular carcinomas<sup>[17,18]</sup>. The NGS-based studies of CRC genomes are summarized in Table 2. These studies identified the unique mutational spectrum and novel targets of genomic alterations in respective cancer types with biological and clinical significance.

## SOMATIC MUTATIONS IN CRC GENOMES

Like other solid tumors, CRC is thought to initiate and progress through a series of genetic and epigenetic alterations. The progression model of colorectal carcinogenesis (*i.e.*, from adenomatous polyp to benign adenoma, eventually progressing to invasive adenocarcinoma) has been referred to as a classical cancer evolution model in

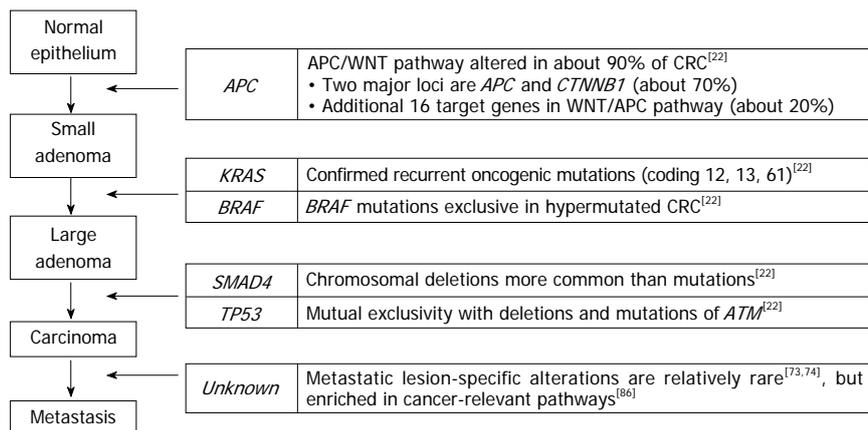


Figure 1 A classical progression model of colorectal carcinogenesis. A classical progression model of colorectal carcinogenesis is illustrated with genes whose alterations are responsible for each of the progressive steps. The right panel shows recent next-generation sequencing-based reports of the corresponding genes. CRC: Colorectal cancer; ATM: Ataxia telangiectasia mutated.

**Table 3 The platforms used in the Cancer Genome Atlas consortium**

Alteration types	Glioblastoma multiforme (2008, TCGA)	Colorectal cancers (2012, TCGA)
Point mutations, indels	Sanger sequencing	Illumina GA and HiSeq DNA Sequencing <sup>1</sup> ABI SOLiD DNA Sequencing <sup>1</sup>
DNA copy numbers	Agilent Human CGH Microarray 244 A Affymetrix Genome-Wide SNP Array 6.0 Illumina Human Infinium 550 K BeadChip	Agilent CGH Microarray Kit 1 × 1 M and 244 A Affymetrix Genome-Wide SNP Array 6.0 Illumina Infinium 550 K and 1M-Duo BeadChip
DNA Methylation	Illumina Infinium DNA Methylation 27	Illumina Infinium DNA Methylation 27 Illumina DNA Methylation Cancer Panel I
Transcriptome	Affymetrix Human Genome U133 Plus 2.0 Agilent 244 K Custom Array Affymetrix Human Exon 1.0 ST Array	Illumina GA and HiSeq RNA sequencing <sup>1</sup> Agilent 244 K Custom Array
MicroRNA	Agilent 8 × 15 K Human miRNA Microarray	Illumina GA and HiSeq miRNA sequencing <sup>1</sup>
Whole-genome sequencing	N/A	Illumina HiSeq DNA sequencing <sup>1</sup>

<sup>1</sup>Next-generation sequencing-based platforms are noted. The platforms used in the genomic characterization of glioblastoma multiforme in 2008 (left) and colorectal cancer genomes in 2012 (right) are shown. TCGA: The Cancer Genome Atlas.

which the CRC genome acquires somatic alterations in a progressive manner throughout several developmental stages. In this model, dysregulation of the APC/WNT pathway *via* the inactivation of *APC* occurs in the normal epithelium as an initiation process, while the loss of *TP53* and *TGF-β/SMAD4* gives rise to clonal expansion of tumor cells in the invasive adenocarcinoma (Figure 1)<sup>[4,19]</sup>. However, the genomic alterations associated with colorectal carcinogenesis may be more complicated than previously assumed. A complete and comprehensive catalogue of oncogenic drivers associated with colorectal carcinogenesis remains to be discovered.

To extend the mutational spectrum in CRC genomes, the first exome-wide screening of approximately 13000 genes was conducted by Sanger sequencing<sup>[20]</sup>. This analysis identified approximately 800 somatic non-silent mutations in 11 CRC genomes. To distinguish oncogenic drivers from neutral passenger mutations, they identified the mutations whose frequency was significantly higher than random. The analysis revealed 69 potential oncogenic driver mutations in CRC genomes, including several well-known cancer-related genes (*i.e.*, *TP53*, *APC*, *KRAS*, *SMAD4*, and *FBXW7*), and a large number of previously uncharacterized genes. Since they examined two distinct tumor types (CRC and breast cancers), they were able to identify the differences in the panel of candidate driver genes as well as

identify the differences in the mutation spectrum between CRC and breast cancer genomes. The difference in the mutation spectrum (*i.e.*, the predominance of C:G to T: A transitions over C:G to G:C transversions in CRC genomes) was confirmed by a subsequent kinase sequencing study across various cancer types<sup>[21]</sup> and by a recent whole-genome sequencing of nine CRC genomes<sup>[16]</sup>.

## NGS-BASED CRC STUDIES - LESSONS FROM THE CANCER GENOME ATLAS CRC STUDY

The advance in sequencing technologies has facilitated the use of genome sequencing for cancer genome studies, including CRC genomes. The largest NGS-based exome sequencing study of CRC genomes to date (approximately 200 CRC genomes) has recently been published as part of the Cancer Genome Atlas (TCGA) projects<sup>[22]</sup>. The platforms used in the multidimensional genomic characterization of CRC genomes were compared with those used for glioblastoma multiforme in 2008 (Table 3)<sup>[23]</sup>. Two important lessons from this large-scale multidimensional TCGA CRC analysis are as follows:

First, similar to previous findings<sup>[20]</sup>, most of the significantly recurrent mutations were observed at

known cancer-related genes, such as *APC*, *TP53*, *KRAS*, *PIK3CA*, *FBXW7*, *SMAD4*, and *NRAS*. The study also revealed frequent coding microsatellite instability (MSI) on *ACVR2A*, *TGFBR2*, *MSH3*, and *MSH6* by manual examination of sequencing reads for 30 known MSI loci. Although the majority of recurrent mutations were previously known, a number of novel mutations were also identified, which may have functional implications on colorectal tumorigenesis. For example, the mutations in *SOX9*<sup>[24]</sup>, *EAM123B*<sup>[25]</sup>, and 14 other genes are known to be implicated in the altered WNT/APC pathway. Although the biallelic inactivation of *APC* and the activating mutation of *CTNNB1* encoding  $\beta$ -catenin are two major events that occurred in about 74% of the total CRC genomes studied, the mutations and deletions of an additional 16 (about 18%) genes in the WNT/APC pathway were not negligible, leading to the conclusion that nearly all CRC genomes (about 92%) have an alteration in the WNT/APC pathway<sup>[22]</sup>.

A study which assigned potential molecular functions to rare mutations in CRC genomes using the pathway-level convergence was previously reported<sup>[26]</sup>. Thus, pathway- or network-level information from available resources (*i.e.*, Gene Ontology<sup>[27]</sup>) and other methodologies to predict the functional impacts of non-synonymous point mutations<sup>[28,29]</sup> may help determine the potential functions of rare mutations and distinguish oncogenic drivers in studies with small-sized cohorts. This issue is also related to the sample-size problem in study design. Due to the limits on sample availability and research budget, many of the cancer mutation studies use a small discovery cohort for the generation of candidate mutations that are subsequently validated in an extended set. Increasing the number of samples in the initial discovery set would be beneficial in identifying events that are not highly recurrent but are still clinically meaningful (*i.e.*, gene fusions involving receptor tyrosine kinases with available inhibitors). For example, the frequency of gene fusions such as *ALK*<sup>[30]</sup> and *RET*<sup>[31]</sup> in lung adenocarcinomas and *FGFR*<sup>[32]</sup> in glioblastoma multiforme are less than 5%. Although the level of recurrence is still the generally accepted functional indicator of genomic alterations<sup>[33,34]</sup>, the incorporation of knowledge from other resources may facilitate the identification of biological or clinically relevant mutations more efficiently in a moderate-sized cohort.

Second, the concordant and discordant relationships between alterations examined across the samples may reveal valuable functional insights. The concordant relationship adopts the concepts of co-expressed networks in which the genes with significantly correlated expression levels (measured by Pearson's correlation coefficients or mutual information) across diverse cellular conditions may have a functional relationship<sup>[35-37]</sup>. Importantly, TCGA-related studies revealed that the exclusivity between the potential oncogenic drivers may be common. For example, TCGA ovarian cancer study showed that the alterations of *BRCA1* and *BRCA2* (including germline or somatic mutations and epigenetic silencing *via* promoter

hypermethylation) are mutually exclusive to each other<sup>[38]</sup>. The method to identify the pairs of exclusive genomic alterations is formulated as a standard analysis pipeline in TCGA projects as Mutual Exclusivity Modules (MEMo) in cancer<sup>[39]</sup>. In CRC genomes, MEMo analysis revealed that nearly half of the TCGA CRC genomes showed an exclusive relationship between the up-regulation of *IGF2* and *IRS2*, and between the mutation of PIK3 pathway genes (*PIK3CA* and *PIK3R1*) and the homozygous deletion of *PTEN*<sup>[22]</sup>. This suggests that the IGF2-IRS2 axis is a major signaling pathway upstream of the PI3K pathway in CRC genomes<sup>[22]</sup>. The mutual exclusivity between the mutations of *TP53* and *ATM* was also identified in the TCGA CRC genomes<sup>[22]</sup>.

## GENOMIC REARRANGEMENTS AND GENE FUSIONS IN CRC GENOMES

Bass *et al.*<sup>[16]</sup> reported whole-genome sequencing (sequencing coverage about 30-fold) of nine CRC genomes. By comparing them with matched normal genomes, they identified approximately 140000 putative somatic mutations per CRC genome, which included approximately 700 non-silent point mutations and indels in coding sequences. One advantage of whole-genome sequencing is that the genome-wide landscape of the mutation spectrum in CRC genomes can be obtained, such as the relative paucity of mutations in exons and higher mutation frequency in intergenic regions than introns. This phenomenon is probably due to the selection pressure and transcription-coupled repair, and is consistent with other types of cancer genomes such as prostate cancers<sup>[40]</sup> and multiple myelomas<sup>[41]</sup>. Since most of the non-synonymous nucleotide substitutions were observed at known cancer genes such as *KRAS*, *APC*, and *TP53*, they focused on novel aspects that can only be identified from paired-end whole-genome sequencing data such as chromosomal rearrangements. Among the approximately 700 candidate rearrangements, 11 events give rise to in-frame fusion genes. The extended screening further revealed that *VTI1A-TCF7L2* fusion is recurrent (3 out of 97 primary CRC genomes) and the siRNA-mediated down-regulation of this fusion transcript reduced the anchorage-independent cell growth *in vitro*, indicative of their potential oncogenic activity.

The low-pass (sequencing coverage approximately 3-4-fold) whole-genome sequencing of 97 TCGA CRC genomes also identified three genomes harboring *NAV2-TCF7L1* fusion<sup>[22]</sup>. The predicted protein structures of fusion proteins lacked the  $\beta$ -catenin binding domain of TCF3 (encoded by *TCF7L1*), which is similar to the fusion of *VTI1A-TCF7L2* that lacks the  $\beta$ -catenin binding domain of TCF4 (encoded by *TCF7L2*). In addition, the inactivation of *TTC28* by genomic rearrangements is of note since this event is recurrent (21 out of 97 cases) and involves multiple partners for rearrangements in TCGA CRC genomes<sup>[22]</sup>. Gene fusions can also be identified from transcriptome sequencing, so called RNA-

seq<sup>[42,43]</sup>. A recent RNA-seq-based study revealed recurrent gene fusions involving R-spondin family members of *RSPO2* and *RSPO3*<sup>[44]</sup>. These fusions were exclusive to *APC* mutations in the observed CRC genomes, suggesting their potential roles in activating the APC/WNT pathway in colorectal carcinogenesis. Cancer-related gene fusion events are gaining attention since there has been no effective gene fusion screening method other than NGS-based paired-end sequencing. More importantly, many of the fusion candidates discovered so far represent oncogenic drivers and clinically actionable events (*i.e.*, the fusion activates potential oncogenes such as tyrosine kinases that can be inhibited by small molecule inhibitors) as shown in recent studies<sup>[30-32]</sup> including the *C2orf44-ALK* fusion in CRC<sup>[45]</sup>.

In addition, it was proposed that the genomic rearrangements in individual cancer genomes can be used as personal cancer markers to trace the disease activity (*i.e.*, to detect recurrences or to evaluate the tumor burden of residual diseases)<sup>[46]</sup>. The proposed method, personalized analysis of rearranged ends, was applied to four cancer genomes, including two CRCs in a pilot test. It was demonstrated that the PCR-based quantification of the rearranged DNA in the plasma correlated well with the treatment course of CRC<sup>[46]</sup>.

## MICROSATELLITE INSTABILITY IN CRC GENOMES

Microsatellites are short tandem repeat sequences present at millions of sites in the human genome<sup>[47]</sup>. MSI defined as the length polymorphism of microsatellite repeat sequences, can arise due to a defect in the DNA mismatch repair system<sup>[48]</sup>. MSI is common in hereditary nonpolyposis colon cancers, also known as Lynch syndrome, where germline mutations of *MLH1* and *MSH2* are commonly observed<sup>[49,50]</sup>. About 15% of sporadic CRC are microsatellite-unstable, where transcriptional silencing of *MLH1* by promoter hypermethylation is common<sup>[51,52]</sup>. The microsatellite-unstable sporadic CRC has distinct clinical and genomic features (*i.e.*, common in right-sided colons and elderly females, and nearly diploid, *etc.*) compared to microsatellite-stable, but aneuploid CRC genomes. The key genes targeted by somatic point mutations and MSI-induced frameshifting mutations are different between the microsatellite-stable and -unstable CRC genomes, as shown in the TCGA CRC study<sup>[22]</sup>. Since the MSI analysis in the TCGA CRC study was limited to a manual search of exome sequencing reads for about 30 known loci with frequent MSI (*i.e.*, *TGFBR2*, *ACVR2A*, *BAX*, *etc.*), a question remains as to whether we can fully exploit the NGS technology to screen the locus-level MSI in an exome- or genome-wide scale. One interesting report by Wang *et al.*<sup>[53]</sup> showed that pancreatic cell lines with a homozygous deletion of *MLH1* (which is a frequent target of promoter hypermethylation in MSI CRC genomes) frequently harbors truncating indels in *TP53* and *TGFBR2*. This suggests that whole genome- or

exome-sequencing data may be used for large-scale MSI screening to identify novel MSI events targeting tumor suppressor genes in cancer genomes.

## NGS AND CRC EPIGENETICS

For decades, DNA methylation has been studied as one of the major cancer-related epigenetic modifications. Until recently, it was recognized that cancer genomes are undermethylated overall, but some genomic loci have focal DNA hypermethylation<sup>[54,55]</sup>. Transcriptional silencing by focal hypermethylation, especially at the CpG islands of gene promoters, is among the putative inactivating mechanisms of tumor suppressor genes in cancer genomes and often preferred over the inactivation by irreversible nucleotide substitutions<sup>[56]</sup>. Yet, the landscape of cancer-associated DNA methylation seems more dynamic than previously anticipated, as revealed by genome-wide CRC methylome studies<sup>[57,58]</sup>. Two recent CRC methylome studies used NGS-based sequencing of bisulfite-treated genomic DNA for bp-resolution methylome profiling<sup>[59,60]</sup>. Both studies proposed the presence of large blocks of DNA hypomethylation that occupied almost half of the genomes. Additionally, they reported that such findings as the genome-wide methylation variability of the adenoma genome is an intermediate between those of normal epithelium and CRC<sup>[60]</sup> and the domains of DNA hypomethylation regionally coincided with those of nuclear lamina attachment<sup>[59]</sup>. In addition, DNA methylation profiling has been also proposed as a means of early CRC diagnosis using non-invasive resources (*i.e.*, blood- or stool-based)<sup>[61,62]</sup>, which can benefit from NGS technologies.

## NOVEL ASPECTS OF CRC GENOMES BY NGS STUDIES

NGS-based genome analysis may facilitate the identification of previously unrecognized, novel features of CRC cancer genomes. For example, owing to its high-throughput nature, NGS analysis may be able to detect the presence of foreign DNA sequences originating from bacterial or viral pathogens. Although the clear association between pathogens and certain human tumor types has been demonstrated in limited cases such as hepatitis B or C viruses with hepatocellular carcinoma, there have been ongoing efforts to use the sequencing data for pathogen discovery<sup>[63,64]</sup>. For instance, Kostic *et al.*<sup>[65,66]</sup> analyzed nine CRC whole genome sequencing data sets<sup>[16]</sup> using their algorithm of PathSeq to identify microbial sequences enriched in CRC genomes compared to those in matched normal genomes. They observed that the sequences of *Fusobacterium* are enriched in CRC genomes, which was also shown in transcriptome sequencing results by independent researchers<sup>[67]</sup>. Although the oncogenic role of *Fusobacterium* in CRC genomes is only beginning to be elucidated<sup>[68]</sup>, these results highlight the possibility that NGS-driven sequencing data will be a valuable resource to identify novel pathogens associated with human cancers.

Chromothripsis is a unique cancer genome-associated phenomenon in which tens to hundreds of chromosomal rearrangements occur in a “one-off” cellular event<sup>[69]</sup>. This phenomenon involves one or a few chromosomes in which massive chromosomal fragmentation is followed by rejoining of the fragments<sup>[70]</sup>. This results in unique genomic signatures that can be identified by paired-end sequencing (*i.e.*, massive intrachromosomal rearrangements in the affected chromosome that can be visualized in a Circos diagram<sup>[71]</sup>) or from copy number profiles (*i.e.*, frequent oscillations between two copy number states indicative of retained and lost chromosomal fragments). After the first discovery of chromothripsis in one chronic lymphocytic leukemia patient by paired-end sequencing<sup>[69]</sup>, Stephens *et al*<sup>[69]</sup> also examined the copy number profiles of 746 cancer cell lines, observing that 2.4% of them (18 cell lines) showed the genomic signatures of chromothripsis. Recently, Kim *et al*<sup>[72]</sup> reported the tumor type-specific frequencies of chromothripsis as measured from a large-scale copy number profile of about 8000 cancer genomes including CRCs. Six out of 366 CRC genomes (1.8%) in the database showed the signature of chromothripsis (*i.e.*, significant frequent alternation between different copy number states<sup>[72]</sup>) and the frequency was not substantially different from the average across the database (1.5%). Of note, Kloosterman *et al*<sup>[73]</sup> reported the paired-end whole-genome sequencing results of four CRC genomes with liver metastases, observing that all cases harbored evidence of chromothripsis. In addition, the comparison between primary and metastatic CRC genomes revealed that most genomic arrangements are shared both by primary and metastatic genomes, indicating that metastasis occurs quite rapidly with few additional mutational events, which was also proposed in mutation-based CRC genome studies<sup>[74]</sup>. Along with chromothripsis, several unique features of cancer genomes have been reported in breast cancer genomes (kataegis; regional hypermutations near rearrangement breakpoints)<sup>[75]</sup> and in prostate cancer genomes (chromoplexy; chains of copy-neutral rearrangements across multiple chromosomes)<sup>[40]</sup>, which may expand the mutational categories in CRC genomes.

## CONCLUSION

We have discussed the recent NGS-based CRC studies in various genomic aspects. The progress of CRC genomic analysis (but not exclusive to CRC) can be summarized into three issues: (1) the screening of clinically actionable targets for personalized targeted medicine; (2) the advancement of pathway-level understanding in colorectal carcinogenesis using a large-scale cohort; and (3) the identification of novel features or mutation types in CRC genomes. In terms of the first issue, Roychowdhury *et al*<sup>[61]</sup> reported an advanced NGS-based cancer patient management protocol that includes low-pass whole-genome, exome, and transcriptome sequencing of cancer genomes. The notable aspects of the protocol are the

timeline (< 4 wk after enrollment) and cost (approximately 3600 USD), as well as the presence of a multidisciplinary sequencing tumor board (STB) to evaluate the mutation profiles of the patients and make a clinical decision. In their pilot study, the STB evaluated the sequencing results from a patient with metastatic CRC harboring *NR4S* mutation and *CDK8* amplification and concluded that BRAF/MEK inhibitors and PI3K and/or CDK inhibitors could be beneficial for the patient. Second, the more complete and comprehensive collection of CRC-related somatic genomic alterations will advance the pathway-level understanding of colorectal carcinogenesis and help distinguish the oncogenic drivers from neutral passengers, as seen in large-scale meta-analyses of cancer genome profiles<sup>[72,77]</sup>. Finally, NGS-driven genomic studies are already reporting novel features of cancer genomes beyond the traditional mutational categories. Besides the MSI and chromothripsis we discussed, some researchers used publicly available genome sequencing data (including those of CRC genomes<sup>[16]</sup>) and reported novel mitochondrial mutations<sup>[78]</sup> and the activity of human retrotranspositions in the cancer genomes<sup>[79]</sup>. Taken together, NGS technology will advance our understanding of CRC genomes and the obtained knowledge will lead to a better diagnosis and personalized targeted therapeutics for CRC management.

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## Gut-lung crosstalk in pulmonary involvement with inflammatory bowel diseases

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### Abstract

Pulmonary abnormalities, dysfunction or hyper-reactivity occurs in association with inflammatory bowel disease (IBD) more frequently than previously recognized. Emerging evidence suggests that subtle inflammation exists in the airways among IBD patients even in the absence of any bronchopulmonary symptoms, and with normal pulmonary functions. The pulmonary impairment is more pronounced in IBD patients with active disease than in those in remission. A growing number of case reports show that the IBD patients develop rapidly progressive respiratory symptoms after colectomy, with failure to isolate bacterial pathogens on repeated sputum culture, and often request oral corticosteroid therapy. All the above evidence indicates that the inflammatory changes in both the intestine and lung during IBD. Clinical or subclinical pulmonary inflammation accompanies the main inflammation of the bowel. Although there are clinical and epidemiological reports of chronic inflammation of the pulmonary and intestinal mucosa in IBD, the detailed mechanisms of pulmonary-intestinal crosstalk remain unknown. The lung has no anatomical connection with the main inflammatory site of the bowel. Why does the inflammatory process shift from the gastrointestinal tract to the airways? The clinical and subclinical pulmonary abnormalities, dysfunction, or hyper-reactivity among IBD patients need further evaluation. Here, we give an overview of the concordance between chronic inflammatory reactions in the airways and the gastrointestinal tract. A better understanding of the possible mechanism of the crosstalk among the distant organs will be beneficial in identifying therapeutic strategies for mucosal inflammatory diseases such as IBD and allergy.

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**Key words:** Inflammatory bowel disease; Pulmonary symptoms; Gut-lung crosstalk; Biao-Li relationship; Social manner

**Core tip:** According to traditional Chinese medicine, the lung and the intestine are a pair of related organ systems (Biao-Li). The lung has no anatomical connection with the main inflammatory site of the bowel. Why does the inflammatory process shift from the gastrointestinal tract to the airways? We hypothesize that each individual cell or molecule not only plays its local role in its own organ, but also plays a "social" role to contribute distal communication through the epithelia. Inflammatory bowel disease may be a good example to study crosstalk between the gut and the lungs.

Wang H, Liu JS, Peng SH, Deng XY, Zhu DM, Javidiparsijani S, Wang GR, Li DQ, Li LX, Wang YC, Luo JM. Gut-lung crosstalk in pulmonary involvement with inflammatory bowel diseases. *World J Gastroenterol* 2013; 19(40): 6794-6804 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6794.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6794>

## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of chronic relapsing and remitting inflammatory bowel diseases (IBDs)<sup>[1-3]</sup>. The incidence of UC and CD is increasing<sup>[4]</sup>. IBDs have become a major gastroenterological problem in developed countries<sup>[4-10]</sup>, and there has been an alarming rise in the traditional low-incidence areas, such as East Asia<sup>[11,12]</sup>, the Indian subcontinent<sup>[13]</sup>, the Middle East<sup>[14]</sup>, Latin America<sup>[15]</sup>, and Eastern Europe<sup>[16,17]</sup>. Although the gastrointestinal tract is the main affected site, both UC and CD are systemic inflammatory disorders that often involve organs other than those of the gastrointestinal tract<sup>[18-23]</sup>. Systemic manifestations can present years after the onset of bowel disease and can affect almost all organs<sup>[18,19]</sup>, including the musculoskeletal<sup>[18,24-26]</sup>, mucocutaneous<sup>[18,19,27,28]</sup>, hepatobiliary<sup>[18,29,30]</sup>, cardiovascular<sup>[18,31-33]</sup>, ocular<sup>[18,34,35]</sup>, renal and genitourinary<sup>[18,36-38]</sup>, pancreatic<sup>[18,29,39-42]</sup>, nervous<sup>[18,43-45]</sup> and bronchopulmonary<sup>[18,19,46-48]</sup> Systems. The extraintestinal manifestations tend to follow the clinical course of IBD and have a high impact on quality of life, morbidity, and even mortality in these patients<sup>[18]</sup>. The reported frequency of extraintestinal syndromes in the patients with IBD varies from 6% to 47%<sup>[18,46-49]</sup>.

Pulmonary involvement among IBD patients was first recognized by Kraft *et al*<sup>[50]</sup> about 40 years ago. Both UC and CD can affect any part of the respiratory system. The spectrum of respiratory disorders occurring among patients with IBD includes small and large airway dysfunction, as well as obstructive and interstitial pulmonary diseases<sup>[18,46-49]</sup>. Screening studies using respiratory symptoms, high-resolution computed tomography (HRCT), bronchoscopy, histological examination, and

pulmonary function tests (PFTs) document some of the earliest changes in airways among the respiratory asymptomatic IBD patients<sup>[18,46-49]</sup>. These changes, especially the subclinical alteration of peripheral airways and parenchymal inflammation may not be detected by routine computed tomography (CT) scan and PFTs<sup>[18,46-49]</sup>. The pulmonary involvements trend to follow the clinical course of IBD<sup>[18,49,51]</sup>. Pulmonary impairment appears more pronounced in the patients with active disease than those with inactive disease<sup>[18,49,51,52]</sup>. The airway inflammation affects quality of life, morbidity, and even mortality among these patients<sup>[18,49,51,52]</sup>. This evidence suggests that local intestinal mucosal inflammation is responsible for the distant airways inflammation<sup>[53-57]</sup>. In a population-based cohort study, patients with chronic obstructive pulmonary disease (COPD) had significantly higher risk for both UC and CD<sup>[58]</sup>. IBD and COPD share many similarities in epidemiological and clinical characteristics, as well as in inflammatory pathology<sup>[59,60]</sup>. Exposure to air pollution may be an important environmental factor for IBD<sup>[61,62]</sup>. There appears to be a direct association between increased environmental pollution and hospitalization among adult IBD patients<sup>[63]</sup>. Inhalation injury is often accompanied by the alimentary tract response<sup>[64,65]</sup>. The local mucosal inflammation in airways may also be responsible for the intestine inflammation and vice versa. In view of the above phenomena, the lungs and intestine are a pair of interacting organ systems<sup>[53-57]</sup>. It is most likely that there are similar inflammatory and immune components of these organs that are the cause of the overlap in pathological changes in respiratory and intestinal mucosal diseases<sup>[59,60]</sup>.

Why the inflammatory process shifts from the gastrointestinal tract to the airways or vice versa remains a mystery. Over the years many explanations for the intestine-lung communication have been proposed, however, no definite conclusions have been drawn. The purpose of this article is to update the plausible hypotheses of intestine-lung axis communication underlying the possible mechanism of pulmonary involvement during IBD pathogenesis.

## PULMONARY INVOLVEMENT IN ASSOCIATION WITH IBD OCCURS MORE FREQUENTLY THAN PREVIOUSLY RECOGNIZED

### *Broad spectrum of respiratory disorders in patients with IBD*

Kraft *et al*<sup>[50]</sup> in 1976 first observed that six patients developed severe, unexplained, chronic bronchopulmonary disease 3-13 years after the onset of nonspecific inflammatory disease of the colon. Since then, various pulmonary manifestations including small and large airway dysfunction or obstruction, inflammation of pulmonary parenchyma and vasculature, as well as bronchopulmonary hyper-reactivity have been reported in IBD<sup>[18,46-48,52,66,67]</sup>.

The spectrum of respiratory disorders occurring among patients with IBD is broad<sup>[18,46-52,68]</sup>. Storch *et al.*<sup>[69]</sup> recommend that primary care physicians must take a broader approach while treating patients with IBD and pulmonary diseases.

### ***Epidemiological evidence to support high prevalence of pulmonary involvement in association with IBD***

Lungs are not generally considered to be involved in IBD. In some studies only a few cases of pulmonary complications have been associated with IBD<sup>[59,67]</sup>, but in others, there is a wide range of pulmonary complications associated with IBD<sup>[18,46-52,67,70]</sup>. Edwards *et al.*<sup>[70]</sup> in a study of 624 IBD patients did not find any association with pulmonary complications. Rodgers *et al.*<sup>[67]</sup> found that there were only three cases of pulmonary complications in their study of 1400 IBD cases. However, other epidemiological investigations and clinical case reports have documented that pulmonary complications occur more frequently in IBD patients than previously recognized<sup>[18,46,47,49,51,52]</sup>. Recently, a large population-based study from Canada indicated that pulmonary complications were the most common concomitant chronic disorder in patients with CD and the second most common in patients with UC<sup>[19]</sup>. The increase in incidence of IBD in the past few decades suggests that environmental factors such as air pollution could contribute to disease pathogenesis<sup>[51-57]</sup>. Association of IBD and polymorphism of many innate immunity genes has been identified<sup>[4,18,53,71,72]</sup>. The high concordance of extraintestinal manifestations in siblings and first-degree relatives with IBD suggests that genetic factors also contribute to the link between intestinal and extraintestinal manifestations<sup>[73]</sup>. The association of pulmonary involvement with IBD appears to be mediated by a complicated interaction of environmental and genetic factors, suggesting there are clear, but undefined, multiple centers of loss of homeostasis in cellular and molecular interactions between the environment and host in both respiratory and gastrointestinal systems.

### ***Latent respiratory abnormalities or dysfunction in IBD patients discovered by HRCT, PFTs and bronchoscopy***

HRCT, sensitive PFTs, bronchoscopy and histological examination have been widely used to discover the earliest pulmonary abnormalities or dysfunction among IBD patients. Many respiratory screening studies using HRCT and PFTs support a high prevalence of clinical and subclinical respiratory abnormalities or dysfunction among IBD patients<sup>[18,46-48,51,74]</sup>. Three studies of randomly selected IBD patients showed incidence rates of pulmonary involvement in 44%, 48%, and 50%, respectively<sup>[47,51,75]</sup>. Although overt pulmonary symptoms may be uncommon, abnormalities on PFT have been noted in > 50% of patients with UC and > 60% of those with CD<sup>[51,76,77]</sup>.

Advances in imaging methods over the past decade have led to an increased understanding of anatomical and physiological pulmonary abnormalities in patients with IBD<sup>[77]</sup>. Even though the majority of patients with pul-

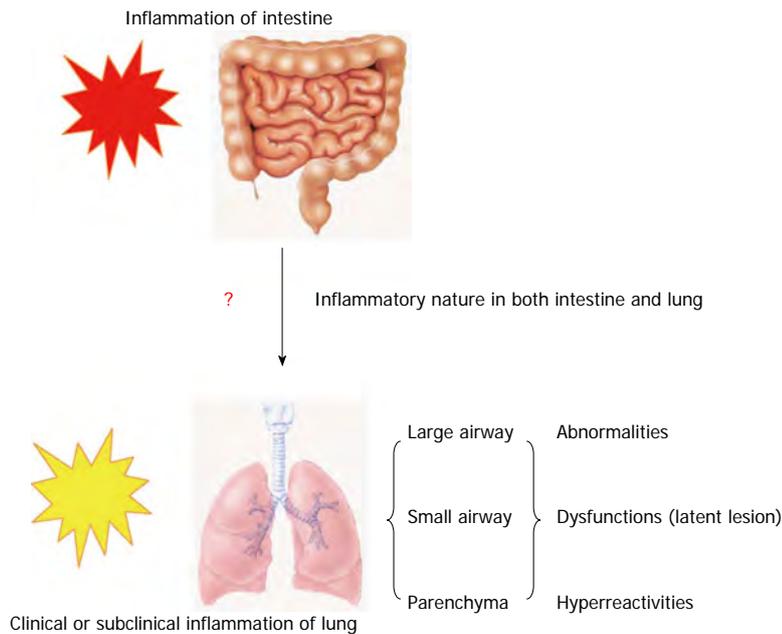
monary abnormalities were asymptomatic, the incidence of at least one abnormality on HRCT of the chest was as high as 39% in UC and 92% in CD patients<sup>[51]</sup>. With the advent of HRCT, it has become obvious that the previously described rarity of pulmonary involvement in IBD is inaccurate<sup>[77]</sup>. HRCT showed changes like bronchiectasis, mosaic perfusion and air trapping, suggestive of obliterative bronchiolitis, and patterns of centrilobular nodules and bronchial linear opacities (“tree in bud” appearance), suggestive of either cellular bronchiolitis or bronchiolectasis with mucoid secretion<sup>[69,78]</sup>. HRCT images also indicate that there are multiple centers of airway responses and mucoid secretion. This suggests that airway epithelium (including goblet cells) has been activated. The response of airway epithelium may be a major contributor to IBD-related lung disease.

Munck *et al.*<sup>[47]</sup> investigated 30 IBD patients (12 with CD, 18 with UC), and found dysfunction of small airways and parenchyma in all IBD patients despite their normal pulmonary physiology. It is likely that the earliest changes occur in the peripheral airways and pulmonary parenchyma, which result in reduced small airways and lung diffusion capacity in all the patients<sup>[49,51]</sup>. Increased disease activity in IBD patients has been shown to be associated with abnormal pulmonary function tests, and suggests a direct pathogenic link<sup>[49,51,79]</sup>.

Bronchoscopy and histological examination from the lung biopsies show changes in the bronchial epithelium, consisting of basal cell hyperplasia, basement membrane thickening, submucosal nonspecific inflammation, small airway fibrosis, and an overall increase in epithelial thickness and granulomatous bronchiolitis<sup>[66,68,79]</sup>. Although the crosstalk mechanism between the bowel and lung remains unknown, the HRCT and histology evaluation show that there is a shift in the inflammatory process from the bowel to the lungs<sup>[49,51,79]</sup>.

### ***Respiratory hyper-responsiveness among IBD patients***

Allergic symptoms, abnormal PFTs and positive skin prick test were more common among the IBD patients compared to the normal population<sup>[28]</sup>. The subclinical nonspecific bronchial hyper-responsiveness, independent of the presence of atopy, in IBD patients who have no other pulmonary symptoms and with normal baseline spirometry, has a high prevalence<sup>[48]</sup>. Colby *et al.*<sup>[80]</sup> reported that the bronchial hyper-reactivity occurred in 48% of patients with UC and CD, even in the absence of any clinical, radiological and functional evidence of airway disease. Bronchial hyper-reactivity occurred in 71% of children and adolescents with CD<sup>[81]</sup>. Allergy is an inappropriate inflammatory reaction to antigen. The allergen triggers an exaggerated immune response, and can aggravate an immediately atopic allergic reaction<sup>[82-84]</sup>. Combination of HRCT and histological examination revealed multiple centers of pulmonary inflammatory responses, which may share many characteristics with atopic allergic reactions<sup>[48,80,81]</sup>. The lungs may duplicate the “social” inflammatory reaction of the intestine (Figure 1).



**Figure 1 Model for inflammatory process in lung and intestine during inflammatory bowel disease.** Clinical or subclinical inflammation in small and large airways and the lung parenchyma accompanies the main inflammation in the bowel during inflammatory bowel disease.

## EPITHELIAL CELLS ARE THE INITIAL AND MAIN TARGET

The epithelial cells are considered not only the frontier sentinels and barriers, but also the central participants in innate and adaptive immune responses during mucosal inflammation<sup>[71,85-88]</sup>. In both the bowel and lungs, these cells provide an important physical barrier to the huge vulnerable biological surface, and are continuously exposed to the external environment. Upon activation, epithelial cells can release large quantities of proinflammatory cytokines, growth factors and chemokines that attract inflammatory cells to initiate and sustain the inflammatory reaction<sup>[71,85-88]</sup>. Direct or indirect interactions of epithelial cells with mast cells, T and B lymphocytes, dendritic cells (DCs), eosinophils, neutrophils, and basophils help to maintain the balance between the environment and the host<sup>[88]</sup>. The airway and gut epithelial cells are at the center of the inflammatory response and play the dominant role in initiation and sustaining the inflammatory reaction<sup>[88]</sup>.

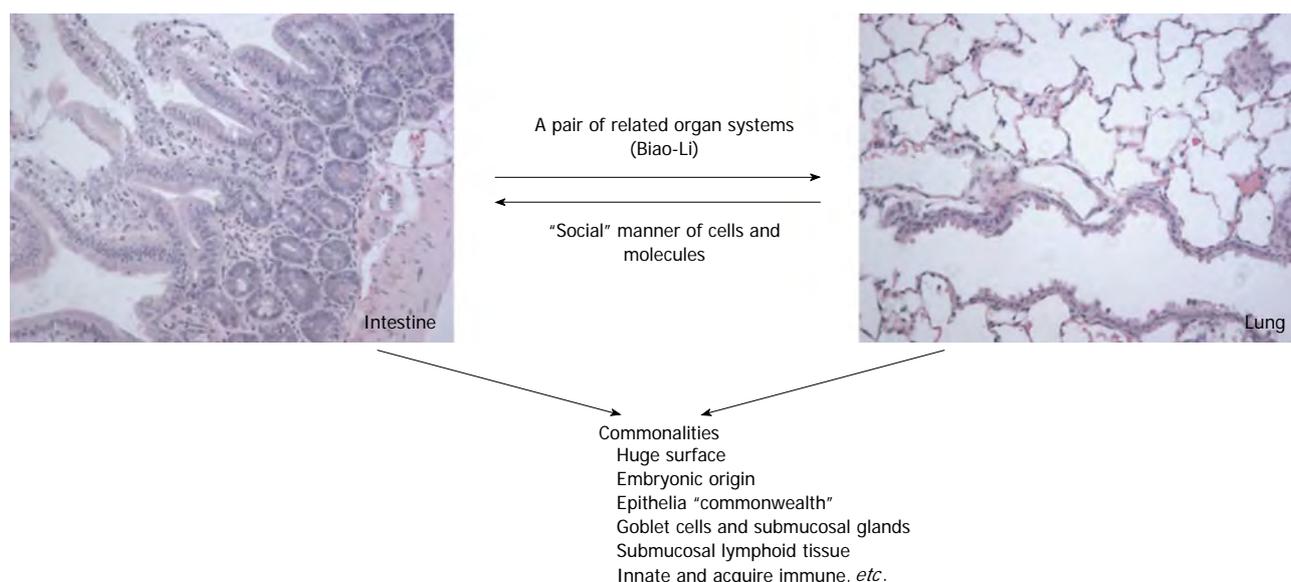
### *Intestinal epithelium is the center of cellular and molecular interaction between host and environment*

The healthy mutual relationship between the microbiota and host depends on the balanced interaction between the microbiota and innate immune activation<sup>[60,71,87,89,90]</sup>. The intestinal barrier plays a crucial role in cellular and molecular interactions between the commensal microflora and possible harmful factors in the intestinal lumen and innate immune system<sup>[60,71,87,89-92]</sup>. The intestinal barrier is composed of a thick mucus layer containing host defense molecules, a monolayer of columnar intestinal epithelial cells, and underlying set of cells (mesenchymal cells, DCs, lymphocytes and macrophages)<sup>[60,71,87,89-92]</sup>. The intestinal epithelial cells are exactly at the center of the

complex system called the intestinal barrier. The intestinal epithelial cells are a single layer of columnar cells consisting of four types of cells: the absorbent enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. It is clear that these four types of epithelial cells present an anatomical and functional barrier to maintain the whole intestinal homeostasis. On the antigen-rich side of the lumen, they secrete and regulate the composition of the thick mucus layer. The intestinal epithelial cells serve as sentinels of innate immunity. The innate immune system is able to recognize bacterial and viral motifs through the toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain families<sup>[93-95]</sup>. The intestinal epithelial cells express several members of the TLRs<sup>[93]</sup>. On the basolateral side, these cells interact and continuously crosstalk with inflammatory cell-rich lamina propria. The intestinal epithelial cells are nonprofessional antigen-presenting cells, which are able to process and present antigens directly to lymphocytes by a highly polarized system with apical antigen sorting, processing and exclusively basolateral presentation. They serve as the major participants in innate and adaptive immune responses<sup>[60,71,87,89-91]</sup>. The intestinal epithelium acts to maintain homeostasis during the steady state, restore the epithelium during insult or injury, and induce inflammatory responses.

### *Airway epithelium is also the center of cellular and molecular interaction between host and environment*

Although initiation of pulmonary inflammation and its association with IBD remains unknown, it is well established that the airway epithelial cells play an important role in the initiation and maintenance of airway inflammation, as well as being the main target<sup>[60,85,88,96-98]</sup>. The airway epithelial cells are also at the center of the airway barrier system because of their anatomical and functional position. On the antigen-rich lumen side, they secrete



**Figure 2 Summary model of plausible mechanisms of lung-intestine communication underlying inflammatory bowel disease-associated pulmonary involvement.** The lungs and intestines are a pair of mutually affected organs. The airways may intrinsically accompany inflammation of the bowel. This distal intrinsic inflammatory response may relate to the common features between the lung and intestine. According to Traditional Chinese Medicine, the lung and intestine have internal and external relationships (Biao-Li). This distal intrinsic inflammatory response may relate to the “social manner” of cells and molecules.

and regulate the composition of the thick mucus layer, while on the basolateral side, they interact and crosstalk with the inflammatory cell-rich lamina propria<sup>[60,85,88,96-98]</sup>. Activated airway epithelial cells can induce synthesis and secretion of immune-cell-mediated host defense molecules such as antimicrobial and antiviral proteins that activate other mucosal innate immune cells<sup>[60,85,88,96-103]</sup>. Activated innate immune responses can secondarily induce the recruitment and activation of DCs and T and B lymphocytes that amplify antigen recognition and antibody production and other adaptive immune responses<sup>[60,96-103]</sup>. Many studies document that the IBD patients develop rapidly progressive respiratory symptoms after colectomy without any identified bacterial pathogens on repeated sputum culture and require oral corticosteroid therapy<sup>[18,66,99,104-106]</sup>, which proves that pulmonary impairment in IBD is not a pathogen-induced inflammation. The pulmonary involvement in IBD may be due to aberrant immunity from the bowel that disrupts airway tissue homeostasis. This aberrant airway epithelial injury/repair may be the mechanism of IBD-related pulmonary involvement<sup>[53-57,107,108]</sup>. Lungs may duplicate atopic inflammation of the bowel. However, the connection between the primary site and the atopic site is unclear. Less attention has been paid to the mechanism of atopic inflammatory reaction that manifests at sites remote from the primary site. The mechanism should be either systemic-factor-mediated cellular and molecular events or some intrinsic connection between the primary and atopic sites. In future, detecting the earliest pulmonary epithelium activation signs or genetic responses during IBD pathogenesis would provide evidence of the linkage between the anatomically disconnected organ systems.

## AIRWAY MAY DUPLICATE THE BOWEL INFLAMMATORY EVENT

Combined HRCT and histological examination have shown that there are multiple centers of airway epithelial (including goblet cells) responses and mucoid secretion in IBD<sup>[78-79,104,109]</sup>. The lung duplicates the inflammatory process of the bowel (Figures 1 and 2). The linkage between the lungs and intestine is hard to understand in the absence of anatomical connections or systemic neuroendocrine immune mediation. Pulmonary inflammation may intrinsically accompany the main inflammation in the bowel, and this distal intrinsic inflammatory response may be related to the “social manner” of cells and molecules, similar to the exaggerated allergen-induced multiple centers of allergy inflammatory response on the surface of the skin, conjunctivae, and airway and digestive tracts<sup>[83-85,88,99,107,108]</sup>.

### *Commonalities between lung and intestine and crosstalk*

There are many commonalities between the lungs and intestines<sup>[18,53,107,108,110,111]</sup>. The gastrointestinal tract and bronchial tree share a common embryological origin that arises from the primitive gut<sup>[10,74,75,77,78]</sup>. Both the lung and intestine possess goblet cells and submucosal glands as part of their luminal structure. In addition, both contain submucosal lymphoid tissue, host defense molecules, and play crucial roles in both innate and acquired immunity<sup>[18,107,108,110,111]</sup>.

In IBD, inflamed bowel mucosa produces many inflammatory mediators and releases them to the circulating system<sup>[4,66,71,86,87,90,102,103]</sup>. The inflamed bowel mucosa also creates the prospect for systemic absorption of

luminal contents, including dietary antigens, digestive enzymes, or specific bacterial products capable of inducing systemic inflammation<sup>[66,102,103]</sup>. Some investigators hypothesize that the pathogenesis of IBD-related airway disease may be associated with circulating inflammatory mediators, specific bacterial products, dietary antigens, or digestive enzymes<sup>[102,103]</sup>. Detectable levels of bacterial lipopolysaccharide and antibodies to bacterial lipid A and peptidoglycan in serum of IBD patients support this theory. However, it is less likely to be accurate in the case of IBD-associated lung disease, because it clearly occurs after colectomy in patients who do not have any ongoing bowel inflammation<sup>[4,66,105,106]</sup>. To the best of our knowledge, none of the circulating inflammatory mediators, dietary antigens, digestive enzymes, or specific bacterial products has been established as being responsible for extraintestinal syndromes during the intestine inflammatory process. In fact, circulating inflammatory mediators are not always enough to drive inflammation from the local mucosa to distal organs. Our previous work suggested that the skin, conjunctivae, airways and digestive tract may join together to fight allergens<sup>[107,108]</sup>. The atopic lesion may duplicate the primary contact site of cellular and molecular events. The atopic march may be due to the intrinsic “social” involvement of the positioned epithelial cells, but may not be totally controlled by the anatomic connection or the circulating systemic factors involved in allergy pathogenesis<sup>[53-57,107,108,112,113]</sup>. Thymic stromal lymphopoietin (TSLP), a general biomarker for skin-barrier defects<sup>[85,107,114]</sup> is an interleukin-7-like cytokine produced by epithelial cells, has emerged as a potential master regulator of both skin and airway inflammation<sup>[85,107,112-115]</sup>. TSLP signaling plays an important role in both airway and skin allergic inflammation. Recently, Zhang *et al.*<sup>[114]</sup> found that increased TSLP expression in skin keratinocytes not only triggers atopic dermatitis-like lesions in skin locally, but also leads to a concomitant ovalbumin (OVA)-induced asthma-like lung inflammation in animals. Furthermore, they elucidated that increased epidermal keratinocyte TSLP expression and subsequent increase in circulating TSLP in a mouse model did not lead to any spontaneous lung inflammation in the absence of OVA sensitization and challenge. High levels of expression of TSLP mRNA in bronchial epithelial cells and submucosa of asthmatic patients<sup>[115]</sup>, suggest that locally produced TSLP could be important for the development and maintenance of asthma<sup>[85,107,112-115]</sup>. TSLP appears to be a crucial factor in driving both skin and lung into inflammation, but solely increased epidermal keratinocyte TSLP expression and subsequent increase in blood circulating TSLP is not sufficient to drive both skin and lung into inflammation<sup>[85,107,112-115]</sup>. The lung inflammation is not triggered by circulating or epidermal TSLP, but requires local TSLP production by pulmonary epithelial cells<sup>[85,107,112-115]</sup>. OVA sensitization and challenge is extremely important to drive both skin and lung into inflammation<sup>[85,107,114]</sup>. The intrinsic connection of skin and lung is through locally produced TSLP after OVA

sensitization and challenge<sup>[85,107,112-114]</sup>.

The plausible mechanism for association of pulmonary involvement with IBD may be the intrinsic airway reactions that accompany the main inflammation in the bowel, as for allergen-triggered, enigmatic, multiple centers of allergic inflammation.

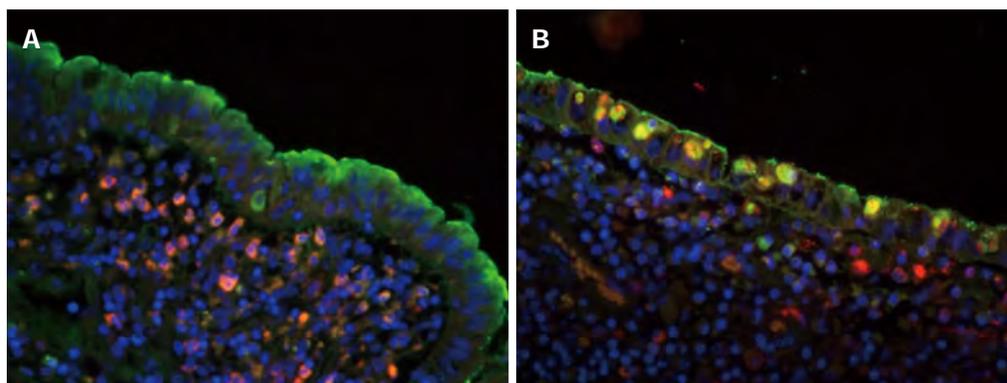
There is clearly communication among organs that share a common embryological origin, or similar cellular and molecular structure without any anatomical interaction<sup>[53-57,65,107,108,110,111]</sup>. Primitive gut has a shared common origin with the gastrointestinal tract, biliary tract, and respiratory system. Our previous work suggested that pulmonary surfactant protein A-like molecule is a frontier host defense protein and that its expression may be associated with some autoimmune diseases<sup>[53,65]</sup>. Latex-mediated allergy is another example of intrinsic activation of non anatomically related organs. During latex-mediated allergy, the skin allergic reaction occurs anywhere in the body and not necessarily at the site of the direct latex contact<sup>[74,75,83]</sup>. How does information transfer from the epidermal keratinocytes at latex-contacted sites to those at uncontacted sites<sup>[107,108,116]</sup>? It is possible that the epidermal keratinocytes at both sites intrinsically communicate because they are close relatives that share common features.

In fact, in IBD, the surface of skin, conjunctivae, and airways is involved in fighting inflammation in the gastrointestinal tract.

As above mentioned, OVA intrinsically drives skin and lung into allergic inflammation by locally produced TSLP<sup>[85,107,112-115]</sup>. Aberrant immune responses to the commensal microbiota in the bowel, which lead to pulmonary inflammation, may be related to some unknown TSLP-like molecules. Detecting these molecules could provide the mechanism of lung-intestine crosstalk.

### ***Pulmonary involvement associated with IBD may be a good example to understand the Biao-Li relationship between lung and intestine***

According to Chinese Medicine, the lung and the intestine are a pair of related organ systems (Biao-Li). The interaction of lung and intestine is mutually affected by internal and external relationships<sup>[47,53-57,107,116-118]</sup>. It means that lung diseases often have colon syndromes and colon diseases may have lung syndromes. The mechanism is complicated, and immunological, environmental and genetic factors should trigger lots cellular and molecular events<sup>[107,108]</sup>. From the pathological findings, the microscopic hallmark of IBD in the intestine and lung is infiltration of chronic inflammatory cells and response of interstitial cells such as fibrocytes and epithelium. In our previous study, we investigated one type of frontier immune cell (CD68-positive macrophages) and one type of frontier host defense molecule [pulmonary surfactant protein (SP)-A], which reside in the lungs and colon. Both frontier immune cell and frontier host defense molecules are overexpressed in inflammatory areas of CD and UC, and in particular, there are many CD68-positive



**Figure 3** Co-localization of surfactant protein-A and CD68 in normal and inflammatory areas. A: Surfactant protein (SP)-A -positive signal was indicated by fluorescence microscopy (green) fluorescence; CD68-positive signal was identified by rhodamine red-X (red) fluorescence; and the cell nucleus was indicated by Hoechst dye (blue). In the normal area, SP-A was located in the surface of the villi, specific epithelium and submucosae, lamina muscularis, mucosae and lymphoid tissues. CD68-positive cells were mainly found in the submucosae, lamina muscularis, mucosae, and lymphoid tissues and in the epithelium; B: In the inflammatory area, CD68-positive cells were dramatically increased in all levels of the bowel wall; especially CD68-positive macrophages in the epithelia of lamina mucosa. The SP-A-positive macrophages were recruited by activated epithelial cells. Double labeled SP-A and CD68 shows that some CD68-positive macrophages expressed SP-A like molecule in the inflammatory bowel disease tissues (original magnification,  $\times 200$ ). Figures reproduced with permission from reference [53].

macrophages among the epithelia of lamina mucosa. The SP-A-positive macrophages are recruited by activated epithelial cells (Figure 3)<sup>[53]</sup>. The epithelium serves as the central participants in innate and adaptive immune responses as well as mucosal inflammation, laboratory evidence of activation of pulmonary epithelium would provide an insight into the “Biao-Li” relationship between lung and intestine during the pathogenesis of IBD associated pulmonary disorders. In view of this, association of pulmonary involvement with IBD may be a good example to understand the Biao-Li relationship between lung and intestine<sup>[53-57,107,116-118]</sup>.

#### *Lung-intestine connection may relate to the “social” property of cells and molecules*

To date, there is a lack of evidence for the participation of any circulating inflammatory mediator, dietary antigens, digestive enzymes, or specific bacterial products as a linkage between the gastrointestinal tract and extraintestinal organs. Both gastrointestinal and airway lesions may be epithelium-mediated chronic inflammation. IBD-associated pulmonary involvement, such as epithelial disease with adoption of a chronic wound scenario, could also be a hypothesis about airway wall and gastrointestinal tract remodeling over the course of the disease<sup>[60,66,80]</sup>. The extraintestinal organs may duplicate “social” inflammation of the gastrointestinal tract. The inflammation of the gastrointestinal tract as a “social” factor drives inflammatory reaction to extra-intestinal organs. There is a clear but undefined communication among organs that share a common embryological origin, or similar cellular and molecular structure despite being located at a distance from each other without any anatomical interaction. The human body originates from a single cell, which makes the whole body a social system. A constant molecular and cellular interaction between the environment and host is required for the establishment and maintenance of homeostasis. Disrupted homeostasis in the gastrointestinal

tract may also affect the airways. Pulmonary involvement associated with IBD as the “social” inflammatory reaction, is a plausible hypothesis. Sharing the common embryological origin or similar cellular and molecular structures may be “closer relatives” in our “social body”. The “social manner” of cell and molecules would also help to attain insight into the pathogenesis of enigmatic complications such as IBD, and the cellular and molecular mechanism of Chinese Traditional medicine.

## CONCLUSION

The frequency of pulmonary abnormalities, dysfunction or hyper-reactivity that occurs in association with IBD is more than previously recognized. More evidence shows that latent inflammation exists in the airways of IBD patients, even without any bronchopulmonary symptoms and with normal pulmonary function. The pulmonary impairment is more pronounced in IBD patients with active disease than in those in remission, and the linkage between lung and intestine may not be easily supported by anatomic connection or systemic neuroendocrine immune mediation. The airways may intrinsically accompany the main inflammation in the bowel. Lung and intestine share a lot of common features. The interaction of lung and intestine is mutually affected by internal and external relationships (Biao-Li). The pulmonary abnormalities, dysfunction or hyper-reactivity in IBD patients may be related to the “social” manner of cells and molecules. Further investigation of epithelium-mediated cellular and molecular events and their interaction in lung and bowel may provide evidence of mutually affected internal and external relationships (Biao-Li) of the lung-intestine axis, and the “social property” of cells and molecules. A better understanding of the mechanism of crosstalk among the distant organs would be beneficial to the elucidation of the etiology and help in identifying therapeutic strategies for mucosal inflammatory diseases such as IBD and

atopic allergic inflammation.

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## Ligation of intersphincteric fistula tract: What is the evidence in a review?

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### Abstract

Broadly, complex fistulas are those that are not low transsphincteric or intersphincteric. The objectives of surgical management are to achieve fistula healing, prevent recurrences and maintain continence. The risk of incontinence associated with treatment ranges from 10% to 57%. The objective of this manuscript is to review the current literature to date on the ligation of the intersphincteric fistula tract procedure (LIFT procedure) as a treatment option in these types of fistula. A search was conducted in Medline, PUBMED, EMBASE and ISI Web of Knowledge, and studies published from January 2009 to May 2013 were included. The primary outcomes were fistula healing rates, mean healing time and patient satisfaction with this surgical technique. Eighteen studies were included in this review. The total number of patients included was 592 (65% male). The median age reported was 42.8 years. The most common type of fistula included was transsphincteric (73.3% of cases). The mean healing rate reported was 74.6%. The risk factors for failure discovered were obesity, smoking, multiple previous surgeries and the length of the fistula tract. The mean healing time was 5.5 wk, and the mean follow-up period was 42.3 wk. The patient satisfaction rates ranged from 72% to

100%. No de novo incontinence developed secondary to the LIFT procedure. There is not enough evidence that variants in the surgical technique achieve better outcomes (Bio-LIFT, LIFT-Plug, LIFT-Plus). This review indicates that the LIFT procedure is primarily effective for transsphincteric fistulas with an overall fistula closure of 74.6% and has a low impact on fecal continence. This procedure produces better outcomes at the first surgical attempt.

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**Key words:** Fistula-in-ano; Ligation; Intersphincteric; Fistula tract; Incontinence; Recurrence; Transsphincteric fistula

**Core tip:** We review the current literature published until today about the ligation of intersphincteric fistula tract procedure. The paper describes the different types of fistulas in which the technique has been used; the cure rates achieved; the reported recurrence rates; types of failures and morbidity related to it. The paper analyzes the prognostic factors for the success; describes the various modifications of the surgical technique and the results obtained with them. The manuscript classifies the types of failures and gives options for their proper treatments. With all these, it sets the achievements and limitations of the technique, with the scientific evidence available today.

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### INTRODUCTION

Perianal abscess and fistulas represent two stages of

the same disease. The main etiology is cryptoglandular. Perianal abscess and fistulas are two of the oldest human surgical entities<sup>[1]</sup>. The objectives of treatment are to achieve fistula healing, prevent recurrences and maintain continence. The risk of incontinence associated with treatment ranges from 10% to 57%<sup>[2]</sup>. The disease has an incidence of 8.6 per 100000 people and nearly 20000 to 25000 fistulas are treated annually in the United States<sup>[1]</sup>. The incidence of fistulae after perianal abscess is 27% to 60%<sup>[3]</sup>. Traditionally, a “complex fistula” is defined by a high risk of recurrence or incontinence following treatment. Broadly, complex fistulas are those that are not low trans-sphincteric or intersphincteric fistulas. The surgical options for these fistulas include fibrin application, plug placement, endorectal advancement flap (ERAF), fistulotomy with primary sphincter repair, partial fistulotomy with seton placement, ultra-low anterior resection and coloanal anastomosis, the ligation of the intersphincteric fistula tract (LIFT) procedure and recently, the video-assisted fistula tract procedure (VAAFT). Trans-sphincteric fistulas comprise 20%-25% of all fistula cases<sup>[3]</sup>. Although plug placement and applying fibrin are still being used, at present, there is a preference for the ERAF and LIFT procedures. The LIFT procedure, which is the topic of this review, involves the following principles: (1) identification of the internal opening; (2) incision at the intersphincteric groove; (3) dissection of the intersphincteric space; (4) identification of the intersphincteric fistula tract; (5) securing ligation and excision of the intersphincteric tract; (6) confirming the removal of correct fistulous tract; (7) opening and curetting the external opening; and (8) closure of the intersphincteric wound. A search was conducted in the Medline, PUBMED, EMBASE and ISI Web of Knowledge databases, using the following terms: LIFT, anal fistula, perianal fistula, fistula-in-ano, rectal fistula, complex anal fistula, ligation of intersphincteric fistula tract, sphincter sparing procedures, clinical trials, outcomes, recurrence, failure, morbidity and incontinence. All of the studies published in the English language from January 2009 to May 2013 were included in this review. We initiated the review from 2009 because the surgical technique was first described in that year. In the studies reported by the same group of authors, the outcomes considered for the analysis were those with a longer follow-up and a larger number of patients<sup>[4-7]</sup>.

## REVIEW OF THE LITERATURE

In the present review, we included 18 papers: eleven were retrospective, two were retrospective and prospective, four were prospective and one was a randomized controlled trial (Table 1). The total number of patients was 592, and 385 were male (65%). The average age reported was 42.82 years. Only a few studies included patients with the following characteristics: rectovaginal fistula, 3 studies with 6 patients in total; cigarette smoking, 2 studies with 21 patients; inflammatory bowel disease, 2 studies; diabetes, 3 studies; HIV, 1 study; and using corticosteroids, 1

study. Other special characteristics mentioned but not numerically specified were the presence of obesity, ischemic heart disease, rheumatoid arthritis and cancer. The most common type of fistula included was trans-sphincteric (73.3%). The percentage of “low” transsphincteric fistulas was 13.5%. The remaining fistulas were classified as horseshoe or hemi horseshoe (48), intersphincteric (11), suprasphincteric (9) and rectovaginal (6). In addition, 34.4% of the population had been previously operated using the same or another surgical technique. The mean operative time reported was 36.16 min. Only two studies reported the length of hospital stay (2.5 d and 1.4 d, respectively), but most of the surgeries were performed on an outpatient basis. The mean healing rate was 74.6% (range: 40%-95%), and the mean healing time was 5.5 wk. The percentage of the population who had a drainage seton before the LIFT procedure was 56% (226/402).

In 2009, Rojanasakul<sup>[8]</sup> first described the technique and reported a success rate of 94%. They included 18 patients, with a recurrence rate of 5.6%.

Bleier *et al*<sup>[4]</sup> conducted a retrospective and prospective trial. They included 39 patients, 51.3% of whom were male. The mean age of the population was 49 years. The average number of previous surgeries to treat perianal fistulas was 2. In addition, 74% of the population had at least one previous failed surgical treatment. The average follow-up period was 20 wk. The success rate was 57%. The latency time to recurrence was 10 wk. Of the total recurrences, 4 recurrences were intersphincteric, 3 were transsphincteric and 1 was a horseshoe type. The incontinence rate was 0%. This represents the first experience in the United States. The same group, recently reported on the treatment of 93 patients (61% male), with a mean age of 43 years<sup>[5]</sup>. A 32% had been previously operated. A drainage seton was placed in 92% of the total patients. The healing rate dropped to 40% with a failure rate of 34%, and 26% of patients suffered a recurrence. The mean recurrence time was 7 mo. Nine patients had a down-staging of the fistula to intersphincteric and were treated with fistulotomy, achieving a secondary healing rate of 57%. The average Wexner score reported was 1. No patient had solid stool incontinence.

Shanwani *et al*<sup>[9]</sup> performed a prospective study. A total of 45 patients were included. In total, 71.1% of patients were male, and the mean age was 41.5 years. The mean operative time was 67.5 min. The average hospital stay was 2.5 d (range: 2-5 d). During an average follow-up period of 9 mo, the cure rate was 82.2%, with an average healing time of 7 wk. The recurrence rate was 17.8%, and the recurrence occurred between 3 and 8 mo after surgery. There were no reported cases of fecal incontinence or morbidity.

There are also variations to the conventional LIFT procedure. Ellis, based on the treatment of rectovaginal fistulas with approximately 92% of success, described the use of a bioprosthetic graft to reinforce the ligation and the closure of the fistula tract, calling it the BioLIFT procedure in a prospective study of 31 patients<sup>[10,11]</sup>. Twenty-

Table 1 Summary of the published articles

Ref.	Year	Study design	n	Age (yr)	Gender	Fistula classification	Preoperative evaluation	Healing rate	Failure or recurrence rate	Morbidity	Incontinence	Follow-up	Comments
Rojanasakul <sup>[8]</sup>	2009	Retro	18	NA	NA	13 LTS 5 HS	NA	94%	R 5.6%	NA	NA	NA	Healing time 4 wk
Bleier <i>et al</i> <sup>[4]</sup>	2010	Retro/ pros	39	49	51.3% male	28 TS, 7 HS, 1 SP, 2 RV	NA	57%	43%	2: Anal fissure and pain	0 (NAO)	20 wk (0-58)	PS mean 3 (0-9) 74% PS R time 10w (2-38)
Shanwani <i>et al</i> <sup>[8]</sup>	2010	Pros	45	41.5 (27-56)	71.1% male	33 TS	44% colonoscopy 48.80% anal USG	82.20%	R 17.7%	0	0 (NAO)	9 mo (2-16)	11.1% PS MOT 67.5 min (35-100) LOS 2.5 d (2-5) Healing time 7 wk (4-10) R time 3-8 mo Bio-LIFT
Ellis <sup>[11]</sup>	2010	Pros	31	48 (30-68)	22 male	31 TS	NA	94%	R 2 (6%)	NA	NA	15 mo (12-30)	Include: CD, CS, DM patients. 100% previous seton 18 PS (PLUG) Satisfaction 100% PS 40%
Ooi <i>et al</i> <sup>[2]</sup>	2011	Retro	25	40 (21-67)	17 male	6 IS, 18 TS, 1 SP	MRI 72%	68%	R 28% (all IS)	0	Basal WS 2 PostOp Global WS 4 HealWS 0	22 wk (3-43)	MOT 39 min (17-100) Healing time 6 wk (3-17) R time 13.5 wk (7-20) Satisfaction 72%
Aboulian <i>et al</i> <sup>[4]</sup>	2011	Retro	25	39	68% male	NA	NA	68%	Fa 32%	2 Vaginal Candidiasis	NA	27 wk (8-158)	Previous seton 65% PS 27%
Sileri <i>et al</i> <sup>[3]</sup>	2011	Pros	18	39 (4-62)	10 male	15 TS 1HS 2RV	100% MRI or anal USG Manometry	83%	R 3 (17%; 1 IS, 2 TS)	1 Thrombosed external hemorrhoid	NA	6 mo (4-10)	EC: CD. Previous seton 100%
Tan <i>et al</i> <sup>[4]</sup>	2011	Retro	93	40 (14-71)	82.8% male	89.2% TS (39 LTS, 44 HTS) 4 IS, 22 HS 6 SP	Anal USG 100%	86%	R 6.4% Fa 7.5% (4 IS, 3 sinus)	NA	NA	23 wk (1-85)	PS 28% Previous seton 17.2%
Wallin <i>et al</i> <sup>[5]</sup>	2012	Retro / Pros	93	43 (21-76)	61% male	16 HS 77 TS	NA	40%	Fa 34% R 26% (Secondary 57%)	NA	WS 1 0%-10% Solid incontinence none (NAO)	19 mo (44-55)	Healing time 4 wk (1-12) R time 22 wk (15-24) Describes types of failures. EC: less 3 mo FU, RV, CD, IAR, LI, PL. 32% PS
Albarian <i>et al</i> <sup>[5]</sup>	2012	Retro	40	43	NA	TS	NA	74%	26% (RFUVA; obesity, CS, PS)	NA	0 (NAO)	18 wk (2-64)	92% Previous seton R time 7 mo (0.8-27) Include: HIV, CD, Obesity, CS, DM.
Tan <i>et al</i> <sup>[6]</sup>	2012	Retro	31 ERAF 24 LIFT	ERAF 49 (19-74); LIFT 41 (16-75)	ERAF 87.1% male; LIFT 87.50% male	High fistulas	100% anal USG	ERAF 93.5%; LIFT 62.5%. P = 0.006	ERAF Fa 6.5%; LIFT Fa 37.5%. P = 0.006	NA	NA	ERAF 6 mo (2-26); LIFT 13 mo (4-67)	Previous Seton 100% EC: VIH, CD. ERAF PS: 58.8%; LIFT PS: 25%

Author	Year	Study Design	n	Age (mo)	Sex	HTS	100% Anal USG or MRI	AH 1 mo	R ERAF 7% vs LIFT 8%, P = NS	LIFT 4% bleeding, 8% dehiscence IS wound. ERAF 7% dehiscence apex	NAO	20 mo	Comorbidities: DM, RA, Ca, IHD (33%) EC: CD
Mushaya <i>et al</i> <sup>[17]</sup>	2012	RCT	39	47.8	LIFT 17 males ERAF 10 males	HTS	100% Anal USG or MRI	ERAF 85% vs LIFT 68% Finally 93% vs 92%	ERAF 7% vs LIFT 8%, P = NS	LIFT 4% bleeding, 8% dehiscence IS wound. ERAF 7% dehiscence apex	Report equal functional outcomes	20 mo	Comorbidities: DM, RA, Ca, IHD (33%) EC: CD MOT LIFT 10 min MOT ERAF 42.5 min Pain less with LIFT Satisfaction LIFT 9.8 vs ERAF 8.1, P < 0.001. RNA favored LIFT (P = 0.016) LIFT-PLUG PS 0 EC: CD, FI, MT, acute abscess; HIV, Tb. MOT 20 min (15-40) Healing time faster PS 10 patients EC: CD, RV Female all healed with first LIFT
Han <i>et al</i> <sup>[18]</sup>	2012	Retro	21	38 (25-56)	19 male	NA	100% Anal USG	95%	NA	0	Mean WS 0 (5% WS 1).	14 mo (12-15)	EC: CD, FI, MT, acute abscess; HIV, Tb. MOT 20 min (15-40) Healing time faster PS 10 patients EC: CD, RV Female all healed with first LIFT
van Onkelen <i>et al</i> <sup>[21]</sup>	2012	Retro	22	45 (17-59)	13 male	All LTS	100% MRI	82% Secondary 100%	0	NA	0 (Rockwood)	19.5 mo (3-35)	EC: CD, RV Female all healed with first LIFT
van Onkelen <i>et al</i> <sup>[22]</sup>	2012	Retro	41	42 (20-69)	32 male	TS	NA	51% Secondary 71%	Fa 20 (8 IS alone)	NA	Rockwood PreOp 10 PostOp 7	15 mo (7-21)	ERAF + LIFT PS 48% 3 previous ERAF EC: RV, CD 68% more 12 mo FU. 18% PS. Previous seton 76%. Length of fistula tract correlates with healing rate. Healing time 8 wk (4-36). F time 4 mo Previous Seton 4 MOT 35 min LOS 1.4 d
Liu <i>et al</i> <sup>[7]</sup>	2013	Retro	38	42	74% male	38 IS	NA	61%	Fa 15 12 early type 3 late type	NA	0 (NAO)	26 mo (3-44)	EC: RV, CD 68% more 12 mo FU. 18% PS. Previous seton 76%. Length of fistula tract correlates with healing rate. Healing time 8 wk (4-36). F time 4 mo Previous Seton 4 MOT 35 min LOS 1.4 d
Lehmann <i>et al</i> <sup>[20]</sup>	2013	Retro	17	49 (30-76)	9 male	All recurrent fistulas. 15 TS (3 LTS, 12 HTS) 2 RV	NA	Complete 47%	40% R 13% Fa	1 Local Hematoma 1 Subcutaneous infection	0 (NAO)	13.5 mo (8-26)	EC: RV, CD 68% more 12 mo FU. 18% PS. Previous seton 76%. Length of fistula tract correlates with healing rate. Healing time 8 wk (4-36). F time 4 mo Previous Seton 4 MOT 35 min LOS 1.4 d
Sirikumpiboon <i>et al</i> <sup>[23]</sup>	2013	Pros	41	40.7	31 male	36 TS (3 LTS, 33 HTS), 4 HS, 1 SP	NA	83% LIFT 83% vs LIFT-plus 85% P = 0.059.	Fa 7 LIFT 4 LIFT-plus 3	LIFT-plus 1 local hemorrhage LIFT 1 Anal fissure	0 (NAO)	19 wk	Healing time 4 wk R time 12 wk

Ca: Cancer; CD: Crohn's disease; CS: Cigarette smoking; DM: Diabetes; EC: Exclusion criteria; Fa: Failure; HIV: Human immunodeficiency syndrome; HS: Horseshoe fistula; HTS: High trans-sphincteric; IAR: Ileo anal reservoir; IHD: Ischemic heart disease; IS: Intersphincteric fistula; LI: Loop ileostomy; LOS: Length of stay; LTS: Low trans-sphincteric fistula; MOT: Mean operative time; NAO: Not assessed objectively; NA: Not available; PL: Patients lost; PostOp: Post-operative; Pros: Prospective; PS: Previous surgeries; R: Recurrence; RA: Rheumatoid arthritis; RCT: Randomized controlled trial; RNA: Resumption normal activities; Retro: Retrospective; RFUVA: Risk factor in univariate analysis; RV: Rectovaginal fistula; SP: Supra-sphincteric fistula; Tb: Tuberculosis; TS: Trans-sphincteric fistula; WS: Wexner score.

two of these patients were men, with an average age of 48 years. The patch was derived from the submucosa of the porcine small intestine with a size of 4 cm × 7 cm, which overlaps the fistula tract for 1-2 cm. Fixation was performed to the puborectalis muscle and to the external anal sphincter with absorbable material. BioLIFT achieved a 94% success rate during an average follow-up period of 15 mo. There were two recurrences (one intersphincteric and one hemi-horseshoe). There was local induration and drainage from the operative wound that resolved without any intervention other than routine postoperative care in 12 patients. The degree of satisfaction reported was 100%.

Ooi *et al*<sup>[12]</sup> conducted a trial including 25 patients (17 males). The mean age was 40 years. Approximately 40% of the patients had been previously operated on, and the preoperative Wexner score of the cohort was 2. The healing rate was 68% with a mean follow-up period of 22 wk. The mean operative time was 39 min. There was no morbidity. The global postoperative Wexner score was 4. In the subgroup of patients who achieved healing, the Wexner score was 0. The mean healing time was 6 wk. All the recurrences (28%) were in the form of intersphincteric fistulas. The authors achieved a 72% patient satisfaction rate.

Aboulian *et al*<sup>[6]</sup> treated 25 patients (68% male) with 26 LIFT procedures. The mean age was 39 years. Of those patients, 65% had been previously operated on by drainage seton placement, and 27% of patients had failed to heal after treatment with another previous fistula technique. An average follow-up period of 27 wk was achieved. The healing rate was 68%. The morbidity reported was low and unrelated to the surgical procedure. In a later report of their series with a larger cohort and longer follow-up, patients who healed completely were contacted every 6 mo thereafter to assess for any recurrence of symptoms<sup>[7]</sup>. This group categorized patients with persistent symptoms or reappearance of symptoms before 6 mo as early failures. Late failures were those with resolution in the early period but return of symptoms after 6 mo. A total number of 38 patients were followed. The mean follow-up period was 26 mo, but 68% of patients had a follow-up period in excess of 12 mo. Only 18% of patients had previous fistula surgery, but 76% had received a drainage seton prior to the LIFT procedure. The study described a 61% healing rate after the first LIFT procedure. A total of 15 patients with failures were reported with a median time to the diagnosis of 4 mo. Of these patients, 12 failures were early type, and 3 failures were late type. Taking into account all failures, 4 were blind infected sinus, 2 occurred in the form of intersphincteric fistula (down-staging effect), and 9 occurred as the same trans-sphincteric fistula. In this series, the median healing time was 8 wk. No incontinence or morbidity was reported.

Sileri *et al*<sup>[13]</sup>, in a prospective study, treated 18 patients (10 of them male), with a mean age of 39 years. All the patients had a history of abscess drainage and seton

placement over a period of 6 to 8 wk. The healing rate was 83% with only 3 recurrences (one was intersphincteric treated with fistulotomy, and the others were 2 transsphincteric fistulas treated with seton placement and ERAF). The average follow-up period was 6 mo. The only morbidity reported was a thrombosed external hemorrhoid.

In one of the largest series described, Tan *et al*<sup>[14]</sup> analyzed the outcomes of 93 patients (82.8% male). The mean age reported was 40 years. Of the patients, 28% had been operated with another surgical technique before, and only 17.2% had a seton drainage for a mean time interval of 11 wk. The average follow-up period was 23 wk. The success rate was 86%, with a mean healing time reported of 4 wk. The recurrence rate was 6.4%, and the failure rate was 7.5%. Of the 7 patients with failure, 4 were down-staged the fistula to intersphincteric and treated with fistulotomy, and 3 patients had a blind sinus treated with silver nitrate and antibiotics. There were 6 recurrent transsphincteric fistulas. The mean time interval between treatment and failure was 22 wk. The authors described three types of failure. Type 1 is a localized failure or blind sinus characterized by secretion or discharge in the intersphincteric wound without evidence of primary opening and adequate granulation of the external orifice. Type 2 is a partial failure with down-staging of the fistula tract (the fistula is now intersphincteric), and type 3 is a total failure with the same previous fistula tract but without involvement of the intersphincteric wound.

Abcarian *et al*<sup>[15]</sup> reported the results of 40 patients with a mean age of 43 years. The cohort had an average of 2 previous surgeries. The healing rate was 74%, but those patients primarily treated with the LIFT procedure had a healing rate of 90%. In contrast, the patients with one previous surgery had a healing rate of 75%, and the patients with two or more previous surgeries had a success rate of 65%. The mean follow-up period was 18 wk. The authors did not report any functional change in continence.

Tan *et al*<sup>[16]</sup>, in a retrospective study, compared endorectal advancement flap (ERAF) *vs* the LIFT procedure after all the patients had been operated on with seton placement. A total of 31 ERAF procedures were performed. The mean age of this population was 49 years (87.1% male). In this group, 58.8% of patients had been previously operated on, and the time interval between seton placement and ERAF was 13 wk. The total healing rate was 93.5%, with an average follow-up period of 6 mo. A total of 24 patients were included in the group treated by the LIFT procedure, with 87.5% of the patients being male and a mean age of 41 years. Only 25% of patients had been previously operated on, and the time interval between seton placement and the LIFT procedure was 14 wk. The mean follow-up period was 13 mo. A success rate of 62.5% was reported. The ERAF procedure was more effective in this study [healing: ERAF (93.5%) *vs* LIFT (62.5%); failure: ERAF (6.5%) *vs*

LIFT (37.5%),  $P = 0.006$ ].

Mushaya *et al*<sup>[17]</sup>, in a randomized and controlled trial, compared the LIFT and ERAF procedures. A total of 39 patients were included with a mean age of 47.8 years. In the LIFT group, there were 25 patients (17 males), and in the ERAF group, there were 14 patients (10 males). The mean follow-up period was 20 mo. The mean operative time of LIFT group was 10 min *vs* 42.5 min in the ERAF group ( $P < 0.001$ ). The postoperative pain was greater in the ERAF group (visual analogue scale: ERAF 1 *vs* LIFT 0,  $P = 0.017$ ). The satisfaction rate favored the LIFT procedure (9.5 *vs* 8.1,  $P < 0.001$ ). The morbidity did not differ between procedures (4% bleeding in LIFT group, 7% partial dehiscence at the apex in ERAF group, and 8% dehiscence at perianal wound in LIFT group). The healing rate at one month was 85% and 68% for the ERAF and LIFT groups, respectively. At the end of the study, the success rates were 93% and 92%, respectively. The recurrence rates were similar (7% in the ERAF group and 8% in the LIFT group,  $P = \text{NS}$ ). The interval between surgery and resumption of daily activities favored the LIFT procedure ( $P = 0.016$ ). The functional outcomes were equal between both techniques.

In another attempt to improve the results, Han *et al*<sup>[18]</sup> described a technique using the insertion of a bioprosthetic anal plug in the fistula tract (LIFT-PLUG procedure). They reported their experience in 21 patients (19 of the male gender), and none had previously received an operation. The mean operative time was 20 min. The healing rate achieved was 95% over an average follow-up period of 14 mo. In this series, the mean healing time of the secondary opening was 2 wk, and at the intersphincteric wound, it was 4 wk (faster than previously reported). There was no morbidity. Only 5% of patients reported a Wexner score of 1. A larger randomized, multicenter prospective trial comparing LIFT-Plug with LIFT is in progress, including selected cases without previous surgeries (clinical trial number NCT01478139)<sup>[19]</sup>.

Lehmann *et al*<sup>[20]</sup> reported the efficacy of the LIFT for recurrent anal fistulas exclusively. They included 17 patients, including 9 males, with a mean age of 49 years. In total, 47% of the fistulas were located posteriorly. Eleven patients had more than two previous surgeries, and six patients had more than 3 surgeries. Only 4 patients had been placed a seton drainage previously (the mean time of latency until the LIFT was 15 mo). The healing rate reported was 76.4%, but only 65% of patients presented complete healing during the mean follow-up period of 13.5 mo. The operative time was 35 min. In addition, 41% of patients received an operation on an outpatient basis with a length of stay in the cohort of 1.4 d. Only 2 complications were reported (local hematoma and subcutaneous infections). In the follow-up, 2 patients developed a recurrence, and 1 patient had a sinus. The complete healing rate was 47%, and the incomplete healing rate was 13% (a total of 60%). In addition, 40% of patients had persistence or recurrent fistula. No *de novo* incontinence was reported.

There are two interesting studies that have attempted to expand the existing indications for the procedure. In the first, van Onkelen *et al*<sup>[21]</sup> described 22 patients who had low transsphincteric fistula. Thirteen patients were male, and the mean age of the cohort was 45 years. Of the 9 female patients, 8 had an anterior fistula, and 10 patients of the cohort had previously received an operation. The healing rate was 82%, with 4 down-stages to an intersphincteric fistula treated by simple lay open. With these patients, the final success rate was 100%. All of the female patients achieved complete healing using the first LIFT procedure. The mean follow-up period was 19.5 mo. There was no fecal incontinence reported (using the Rockwood fecal incontinence severity index). The same group raises the possibility of use in conjunction the ERAF and LIFT procedure to prevent recurrence due to infection at the residual tissue<sup>[22]</sup>. The researchers analyzed the results of a series with 41 patients (32 of them male). The mean age was 42 years. In total, 48% had received previous operations (3 ERAF procedures). The LIFT procedure was performed first followed by the ERAF. A healing rate of 51% was reported with a mean follow-up period of 15 mo. Of the failures, 12 of the failures had drainage in the external opening and the intersphincteric wound, and only 8 had drainage in the intersphincteric wound alone. This subgroup of patients was treated by lay open fistulotomy with a secondary healing rate of 71%.

With a new modification of the surgical technique, Sirikurnpiboon *et al*<sup>[23]</sup> compared the effectiveness of adding a partial fistulotomy until the external sphincter (called the LIFT-PLUS procedure) in a prospective study of 41 patients. A total of 20 patients underwent the LIFT procedure (with only curettage of the tract and widening of the external opening), and 21 underwent the LIFT-plus procedure. The average age of the population was 40.7 years. The healing rate achieved was 83%, with a mean follow-up period of 19 wk. The median wound healing time was 4 wk, and the mean time to recurrence was 12 wk. There was no incontinence reported. Morbidity cases included one anal fissure and one local bleeding in the LIFT-plus group and one anal fissure in the regular procedure group. There were 7 treatment failures: 4 in the LIFT group (3 recurrences and 1 sinus abscess) and 3 in the LIFT-plus group (2 recurrences and 1 intersphincteric fistula). All of these patients were healed using the same technique without morbidity or change in continence status. The healing rate by group was 81% in the LIFT-procedure and 85% in the LIFT-plus group, respectively ( $P = 0.0529$ ).

Lastly, a patient who underwent stapled hemorrhoid-ectomy and subsequently developed a remnant sinus tract that was successfully treated with the LIFT procedure<sup>[24]</sup>.

## COMPARISON OF THE RESULTS

In 1993, Matos *et al*<sup>[25]</sup> described a technique of excision of intersphincteric anal gland infection. They excised the

entire fistula tract, in addition to primary repair, by means of an intersphincteric approach by suturing the internal anal sphincter defect. Their success with 20 patients was only 45%. These poor results were attributed to blood supply issues that resulted in wound breakdown.

In the two studies comparing the ERAF against LIFT procedure, the results reported effectiveness of 94% *vs* 62.5% and 93% *vs* 92%, respectively<sup>[16,17]</sup>. In the former study, the follow-up was shorter for the ERAF group<sup>[16]</sup>. It is also important is the larger proportion of patients with previous fistula surgeries in the ERAF group, a possible selection bias. The reported success rate of the ERAF group was unusually higher than previously reported in other trials<sup>[26,27]</sup>. In these previous studies, the authors did not objectively evaluate functional outcomes. Nevertheless, there have been reports citing incontinence rates of up to 35% after ERAF<sup>[28]</sup>.

Not all studies specifically stated the types of fistulas treated. Although the procedure is theoretically ideal for high transsphincteric fistulas, the most common type of fistula was transsphincteric. In addition, van Onkelen *et al*<sup>[21]</sup> described the results of the procedure in the treatment of low transsphincteric fistulas. They reported a final and secondary healing rate of 100% without effect on continence. Bokhari *et al*<sup>[29]</sup> reported that major and minor incontinence after fistulotomy for low fistulas reached up to 5% and 11%, respectively. These authors noted that the other factors taken into account for a greater risk of incontinence are female sex and anterior fistulas or at-risk for obstetric history<sup>[29]</sup>. Garcia-Aguilar *et al*<sup>[30]</sup> reported major and minor incontinence after fistulotomy for low transsphincteric fistulas in 44% of their patients. They also observed that female sex and an internal opening located in the midline anteriorly were predictive factors of impaired continence after fistulotomy<sup>[30]</sup>. Cavanaugh *et al*<sup>[31]</sup> demonstrated that only the amount of external anal sphincter divided correlated with fecal incontinence in severity index scores. It appears possible that the division of the lower part of the external anal sphincter can be avoided in the treatment of transsphincteric fistula using the LIFT procedure.

Two studies reported the routine ligation of the primary internal orifice in their application of the LIFT technique<sup>[5,6]</sup>. During the LIFT procedure, Wallin *et al*<sup>[5]</sup> no-touched the primary opening in 87% of cases, ligated them in 8% of cases, performed a partial internal sphincterotomy in 4% of cases, used Alloderm<sup>®</sup> in 5% of cases and created a mucosal flap in 1% of cases. In a univariate analysis, only the use of biologic mesh displayed a tendency for healing, but the proportions of patients were scarce and did not reach statistical significance. In the study written by Aboulain *et al*<sup>[6]</sup>, the primary internal opening was closed in the mucosal side within the anal canal to prevent the entry of new infective agents. They achieved a healing rate of 68%.

Specifically, the use of a bioprosthetic mesh (Bio-LIFT procedure) reported a 94% success rate, and the use of a plug (LIFT-PLUG procedure) resulted in a reported

cure rate of 95%<sup>[11,18]</sup>. The BioLIFT technique has two potential disadvantages. First, it requires a more extensive dissection in the intersphincteric space. The physiologic consequences of this dissection have not been studied and are unknown. The second disadvantage of both techniques is the relatively high cost of the bioprosthetic materials. The healing time in the study that used the PLUG was 2 wk for the secondary external orifice and 4 wk for the intersphincteric wound (faster than previously reported)<sup>[18]</sup>. These series do not conclusively demonstrate a benefit that would justify the increased cost of the use of a bioprosthetic material. The addition of the partial excision of the fistula tract (partial fistulotomy) until the external anal sphincter is reached (LIFT-plus), or the use of both techniques (LIFT and ERAF procedures) simultaneously in the same patients, did not display any advantage<sup>[22,23]</sup>. To date, there have been no prospective randomized trials comparing the modifications made to the original technique.

Although most studies include a high percentage of previously treated patients, the results in a series of patients with only recurrent fistulas indicated a cure rate of 47%. The scarring following the resolution of the inflammatory post-surgical response can result in fibrosis and obliteration of the intersphincteric space. This makes the dissection in the intersphincteric plane difficult. Tan *et al*<sup>[6]</sup> concluded that given the simplicity of the LIFT procedure, clinicians should still perform the LIFT procedure in patients presenting for the first time and recommend the ERAF procedure in patients with multiple previous surgeries and a scarred perianal region.

Only one trial classified the therapeutic failures as early (80%) and late (20%)<sup>[7]</sup>. In this trial, Aboulain *et al*<sup>[6]</sup> recommended that in patients with persistent symptoms it may be prudent to observe and manage symptoms with local care up to 6 mo before planning for additional treatment. The researchers affirmed that it is important to individualize all cases because some patients may require earlier intervention if their symptoms worsen or develop significant sepsis<sup>[6,7]</sup>. Tan *et al*<sup>[6]</sup> considered that meticulous dissection along the intersphincteric plane while maintaining the integrity of the internal sphincter and the anal mucosa is critical. Any breach or button-hole of the anal canal mucosa during the procedure can lead to a higher risk of failure. Taking into account the classification previously described for recurrences and the results of 12 studies, nine cases were type 1 (blind sinus), thirty-two cases were type 2 (intersphincteric fistula) and forty-seven cases were type 3 (transsphincteric fistula)<sup>[4-7,12-14,16,17,21-23]</sup>. The recommended treatments are local measures for type-1 failures, fistulotomy for type-2 failures, and reperforming the LIFT procedure or ERAF procedures for type-3 recurrences.

The risk factors for failure were obesity, smoking, multiple previous surgeries and the length of the fistula track<sup>[7,15]</sup>. In a retrospective study, the healing rate for patients without previous surgery was 95%, whereas the rate for those with multiple surgeries was 65%<sup>[15]</sup>. A pre-

viously unreported finding was that for every one centimeter increase in fistula length, the odds ratio for healing decreased by 0.55 (95%CI: 0.34-0.88,  $P = 0.01$ ). In this study, the median length of fistula tract was shorter in the healed group compared with the failed group (4 cm *vs* 6 cm,  $P = 0.004$ ). After choosing 3 cm as an arbitrary cutoff point, fistula tracts under three centimeters had significantly higher primary healing (85% *vs* 48%,  $P = 0.04$ ). In addition, 66% of this cohort had a tract length of more than 3 cm<sup>[7]</sup>. van Onkelen *et al*<sup>[22]</sup> reported that a past history of previous surgeries, seton placement, lateral localization of primary opening and horseshoe extension showed a trend for recurrence. Sirikurnpiboon *et al*<sup>[23]</sup> described that body mass index was the only predictor factor for failure in a univariate analysis. Failure to identify the fistula tract occurred more often in obese patients with a body mass index of more than 30 kg/m<sup>2</sup> ( $P = 0.001$ ).

There were fourteen complications, which included anal fissures (4), bleeding (3), intersphincteric wound dehiscence (2), vaginal candidiasis (2), chronic anal pain (1), a thrombosed external hemorrhoid (1), and a subcutaneous infection (1). All these were mild and resolved with conservative treatment. It is important to mention that only four trials used a standardized scale to assess functional outcomes<sup>[12,18,21,22]</sup>. In summary, no de novo incontinence developed secondary to the LIFT procedure with an overall follow-up period of 42.3 wk.

Only 10 of 18 studies reported the use of a seton before LIFT procedure<sup>[5,7,11,13,14,16,17,20-22]</sup>. For the LIFT procedure to be effective, an epithelialized well-formed tract is advised. In theory, if the tract is inflamed or in the absence of enough granulation tissue, there may not be adequate tissue strength to permit ligation. However, Mitalas *et al*<sup>[32]</sup>, found no correlation between prior seton drainage and the presence of epithelium. None of the studies in this review indicated a benefit in using a seton before LIFT procedure.

## CONCLUSION

The currently available information indicates that the LIFT procedure is a feasible and effective surgical technique, with low impact on fecal continence. Its main indication is for transsphincteric fistulas in patients without previous surgery and with short fistula tracts. Patients with more complex fistulas, especially with multiple previous surgeries, should be considered for the ERAF procedure. There is a lack of evidence to recommend the combined use of prosthetic materials or to perform the combined LIFT-ERAF procedure. Further randomized controlled trials are needed to recommend routinely the LIFT procedure against other surgical techniques for anal fistulas.

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## Curcumin cytotoxicity is enhanced by PTEN disruption in colorectal cancer cells

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### Abstract

**AIM:** To investigate the effects of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) deficiency on the cytotoxicity of chemotherapeutic agents toward colorectal cancer cells.

**METHODS:** PTEN-deficient colorectal cancer (CRC) cells were generated by human somatic cell gene targeting using the adeno-associated virus system. The cytotoxic effects of compounds including curcumin, 5-fluorouracil (5-FU), dihydroartemisinin (DHA), irinotecan (CPT-11) and oxaliplatin (OXA) on cancer cells were determined using the MTT assay. Enhanced cytotoxicity of curcumin in PTEN-deficient CRC cells was observed, and this was confirmed using clonogenic assays. Apoptosis and cell cycle progression were analyzed by flow cytometry.

Levels of apoptosis and cell cycle-related proteins were examined by Western blotting.

**RESULTS:** We developed an isogenic set of CRC cell lines that differed only in their PTEN status. Using this set of cell lines, we found that disruption of the *PTEN* gene had no effect on the sensitivity of CRC cells to 5-FU, CPT-11, DHA, or OXA, whereas *PTEN* disruption increased the sensitivity of CRC cells to curcumin. Loss of PTEN did not alter the curcumin-induced apoptosis in CRC cells. However, PTEN deficiency led to an altered pattern of curcumin-mediated cell cycle arrest. In HCT116 *PTEN*<sup>+/+</sup> cells, curcumin caused a G2/M phase arrest, whereas it caused a G0/G1 phase arrest in HCT116 *PTEN*<sup>-/-</sup> cells. Levels of cell cycle-related proteins were consistent with these respective patterns of cell cycle arrest.

**CONCLUSION:** Curcumin shows enhanced cytotoxicity toward PTEN-deficient cancer cells, suggesting that it might be a potential chemotherapeutic agent for cancers harboring PTEN mutations.

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**Key words:** Phosphatase and tensin homolog deleted on chromosome 10; Curcumin; Chemotherapeutic agents; Cell cycle; AKT signaling

**Core tip:** Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) mutations lead to cancer progression and drug resistance. Chemotherapeutic agents with enhanced effectiveness against cancers with PTEN mutations are urgently required. In this study, we generated an isogenic set of human colorectal cancer cell lines that differed only in their PTEN status. We found that curcumin showed enhanced cytotoxicity in cancer cells deficient in PTEN. Importantly, PTEN deficiency led to an alteration in the pattern of curcumin-induced cell cycle arrest, which was associated with the PTEN/AKT/p21

pathway. Our findings suggest that curcumin is a potential chemotherapeutic agent for PTEN-mutant cancers.

Chen L, Li WF, Wang HX, Zhao HN, Tang JJ, Wu CJ, Lu LT, Liao WQ, Lu XC. Curcumin cytotoxicity is enhanced by PTEN disruption in colorectal cancer cells. *World J Gastroenterol* 2013; 19(40): 6814-6824 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6814.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6814>

## INTRODUCTION

Cancer is a leading cause of death worldwide and increasing attention has been focused on the strategies to reduce its incidence<sup>[1]</sup>. Chemotherapy is one of the main means of treating cancers; however, resistance to chemotherapeutic drugs remains a major obstacle to effective cancer therapy<sup>[2,3]</sup>. Therefore, novel intervention strategies to enhance the effectiveness of chemotherapeutic drugs and reduce their resistance are urgently required.

Cancer is caused by a series of genetic changes, including mutations in oncogenes and tumor suppressor genes<sup>[4]</sup>. Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is one of the most frequently mutated tumor suppressor genes in human cancers, after *p53*<sup>[5]</sup>. *PTEN* is involved in many important biological processes, including cell proliferation, growth, migration and death<sup>[6]</sup>. Germline mutations of *PTEN* result in several rare cancer predisposition syndromes, such as Cowden disease, Bannayan-Zonana syndrome, Proteus syndrome and Lhermitte-Duclos disease<sup>[7]</sup>. Mice heterozygous for *PTEN* develop spontaneous tumors in a number of organs<sup>[8]</sup>. Conditional deletion of the murine *PTEN* gene leads to tissue-specific tumorigenesis<sup>[9]</sup>. *PTEN* acts as a lipid phosphatase that antagonizes the phosphatidylinositol 3-kinase (PI3K) signaling by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) back to phosphatidylinositol (4,5)-bisphosphate (PIP2). Mutations in *PTEN* lead to constitutive activation of AKT kinase and other downstream effectors, and thus promote tumorigenesis<sup>[10]</sup>.

*PTEN* mutations are found in a number of human cancers, including cancers of the colon, breast, lung, liver, and lymphatic system<sup>[11-15]</sup>. The fact that mutated *PTEN* is present only in the cancer cells makes *PTEN* an appealing drug target for cancer treatment<sup>[16]</sup>. However, mutational inactivation of *PTEN* leads to cancer progression and chemotherapy resistance. Loss of *PTEN* expression is frequently observed in trastuzumab or tamoxifen-resistant breast cancers<sup>[17,18]</sup>. Drug resistance induced by *PTEN* deficiency is also observed in colorectal cancer (CRC). Loss of *PTEN* occurs in 35% of CRC<sup>[19]</sup>, and *PTEN*-deficient CRC cells are more resistant to cetuximab than *PTEN*-expressing cells<sup>[20]</sup>. In addition, loss of *PTEN* expression results in poor clinical outcome in CRCs treated with anti-EGFR therapy<sup>[21]</sup>. To identify

chemotherapeutic agents that could overcome resistance in *PTEN*-mutant CRC, we developed an isogenic set of human CRC cell lines that differed only in their *PTEN* status. Cytotoxicity analysis of these cell lines with several anti-cancer agents showed that curcumin had enhanced cytotoxicity towards CRC cells deficient in *PTEN*. Moreover, loss of *PTEN* expression led to a change in curcumin-induced cell cycle arrest patterns, which might be associated with *PTEN*-regulated AKT/p21 signaling. Our findings suggest that curcumin might have potential in the treatment of cancers with *PTEN* mutations.

## MATERIALS AND METHODS

### Cell culture

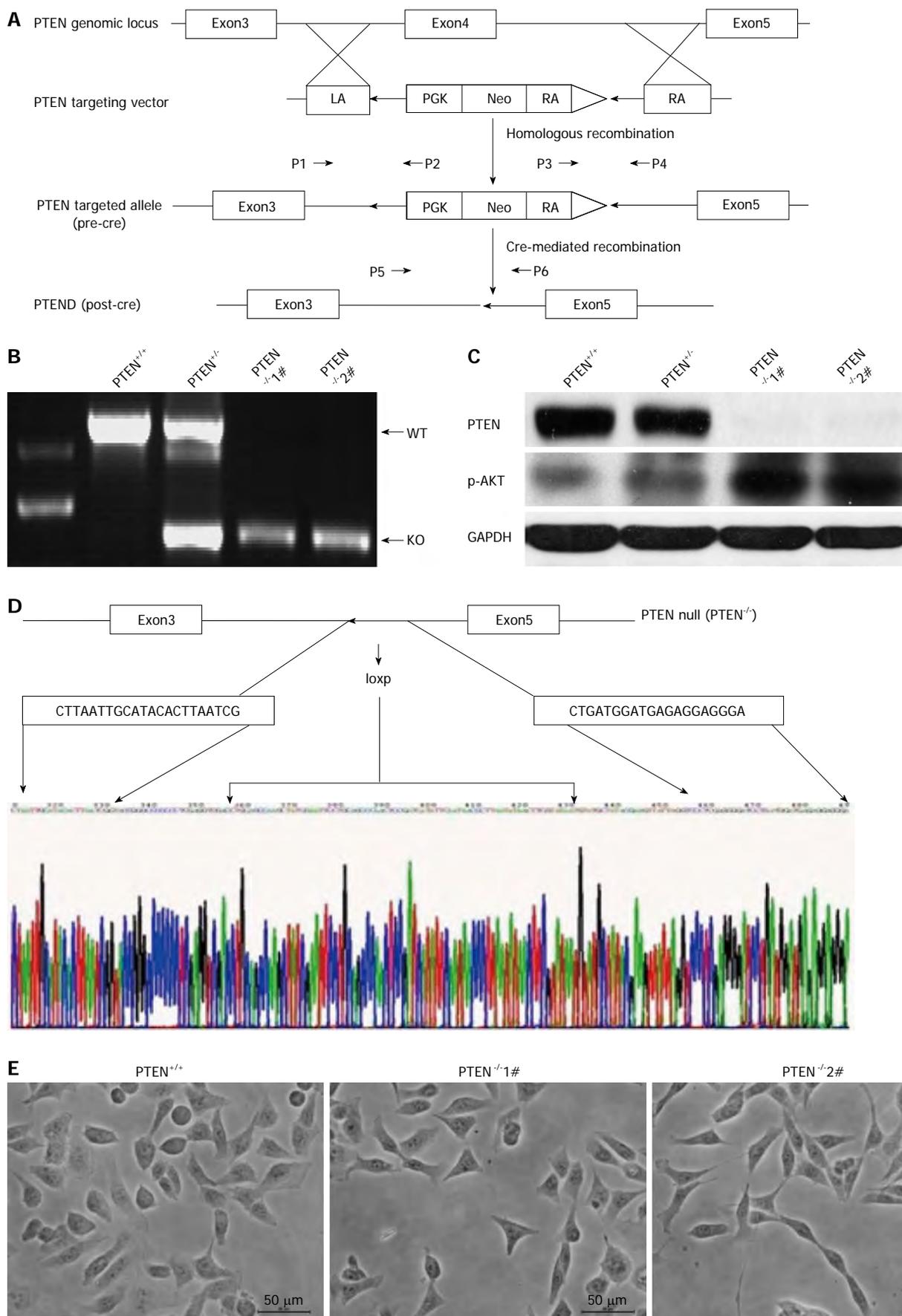
Human colon cancer HCT116 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, United States). HCT116 *p53*<sup>-/-</sup> cells were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD, United States). The cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. Cells harboring the targeting vector were grown in medium containing 1000 µg/mL G418.

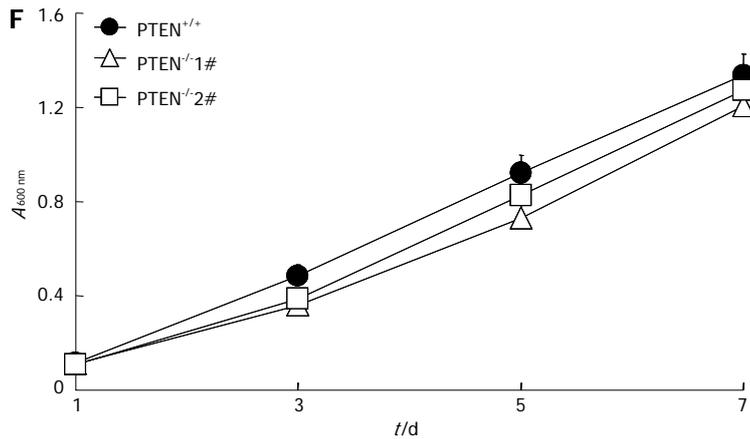
### Reagents and antibodies

Curcumin, 5-fluorouracil (5-FU), dihydroartemisinin (DHA), irinotecan (CPT-11), and oxaliplatin (OXA) were purchased from Sigma-Aldrich. *PTEN*, AKT (pan), phosphorylated AKT (p-AKT), p53, caspase 3, caspase 9, GAPDH, and beta-actin antibodies were obtained from Cell Signaling Technology. Bcl2, p21, p27, Cyclin B1, Cdc2, Cyclin D1, PARP and peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology.

### Gene targeting in HCT116 cells

Human somatic cell gene targeting was performed using adeno-associated virus vectors. A *PTEN*-targeting vector was constructed to delete exon 4 of the *PTEN* gene in HCT116 cells. To create this vector, homology arms were amplified from a human genomic DNA template by polymerase chain reaction (PCR), sequentially cloned into the pMD18-T vector (TaKaRa) and sequenced. The constructed targeting vector was co-transfected with pHelper and pAAV-RC plasmids into HEK293 cells to obtain a *PTEN*-AAV viral stock that was used to infect HCT116 cells. After infection with recombinant virus, HCT116 cells were seeded in 96-well plates and selected with 1000 µg/mL G418 for 2 wk. Individual clones were obtained, expanded, cryopreserved, and tested by PCR for the presence of a heterozygous knockout. Heterozygous clones were transfected with the pCX-CRE plasmid, and then plated at a density of 200 cells/well in a 96-well plate to obtain single cell-derived clones. These clones were then screened by PCR to identify those in which the PGK-neo cassette had been deleted. Then the *PTEN*<sup>+/-</sup> cells were re-transfected with the *PTEN*-AAV virus, to obtain





**Figure 1 Gene targeting at the *PTEN* locus.** A: Schematic illustration of the strategy used to inactivate phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) by somatic cell gene targeting. The primers (P1, P2, P3, P4, P5, and P6) used for polymerase chain reaction (PCR)-based genotyping are indicated; B: Identification of the desired targeted clones by PCR. Primers P5 and P6 were used to amplify a 1,155-bp product from the wild-type *PTEN* allele and a 522-bp product from the final knockout allele, respectively; C: Western blotting analysis of *PTEN* expression; D: Confirmation of *PTEN* exon 4 deletion in *PTEN*-deficient cells by sequencing; E: Morphology of *PTEN*<sup>+/+</sup> and *PTEN*<sup>-</sup> cells. F: Determination of cell viability for *PTEN*<sup>+/+</sup> and *PTEN*<sup>-</sup> cell lines using the MTT assay. *PTEN*<sup>-1#</sup> and *PTEN*<sup>-2#</sup> are two *PTEN* knockout clones. LA: Left arm; RA: Right arm; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

clones in which both *PTEN* alleles had been targeted.

### MTT assays

Cell viability was determined using the MTT assay, as described previously<sup>[22]</sup>. Briefly, *PTEN*<sup>+/+</sup> and *PTEN*<sup>-</sup> cells were seeded in 96-well plates in triplicate. At the indicated time points, 10  $\mu$ L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) stock solution (5 mg/mL) was added to each well. Plates were incubated at 37 °C for another 4 h. The medium was carefully removed and the formazan produced was dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at a wavelength of 570 nm on a microplate reader (BioRad Model 550). To test the compounds for cytotoxic effects, cells were seeded at a density of  $1 \times 10^4$  cells/well in 96-well plates and cultured for 24 h. One-hundred microliters of medium supplemented with twice the desired concentration of compounds was then added to each well. After incubation for 72 h, MTT solution was added. Four hours later, formazan production was measured as described earlier. The viability was calculated as % viability = (OD of treated cells/OD of control cells)  $\times$  100.

### Clonogenic assays

A total of 1000 cells were plated in 6-well plates, in triplicate, and incubated for 24 h to allow cells to adhere. After treatment with 10- $\mu$ mol/L curcumin or 0.1% DMSO for 48 h, cells were carefully washed and drug-free medium was added. The plates were incubated at 37 °C for 10-12 d and stained with 0.5% (w/v) crystal violet. Colonies containing more than 50 cells were scored as positive. The experiment was performed at least three times using triplicate cultures.

### Apoptosis analysis

Curcumin-induced apoptosis was quantified by flow cy-

tometry using the Annexin V-FITC Apoptosis Detection kit according to the manufacturer's instructions. Briefly,  $5 \times 10^5$  cells were seeded in 6-well plates and treated with 10- $\mu$ mol/L curcumin or 0.1% DMSO for 48 h prior to analysis. Floating and trypsinized adherent cells were collected, washed with PBS, and resuspended in cold binding buffer. After incubation for 15 min in the dark at 4 °C, 10  $\mu$ L of Annexin V-FITC and 5  $\mu$ L of propidium iodide (PI) solution were added. Samples were analyzed using an FACS-Calibur cytometer (Becton Dickinson).

### Cell cycle analysis

A total of  $5 \times 10^5$  cells were seeded in 6-well plates and treated with 10- $\mu$ mol/L curcumin or 0.1% DMSO for 48 h. Then, the cells were harvested by centrifugation at 1000 rpm for 5 min. Cell pellets were washed twice with PBS, fixed with ice-cold 70% ethanol and stored at -20 °C overnight. Then, the pellets were washed with cold PBS, suspended in 500 mL PBS containing 50 mg/mL PI, 0.1 mg/mL RNase A and 0.05% Triton X-100, and incubated at 37 °C for 40 min in the dark. The cell cycle distribution was determined by flow cytometry (FACSCalibur; Becton Dickinson). The experiment was repeated thrice under the same conditions.

### Western blotting analysis

Cells were grown to 90% confluence in a 6-well plate. After washing twice with PBS, cells were resuspended and lysed in cold lysis buffer (50 mol/L Tris/HCl, pH 7.4, 5 mol/L EDTA, 0.5% NP-40, 150 mol/L NaCl) supplemented with the Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology). After incubation on ice for 20 min, the lysate was centrifuged at 12000 rpm for 20 min at 4 °C, and the supernatant was collected. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, United States). Equal amounts of total protein were separated by SDS-PAGE and trans-

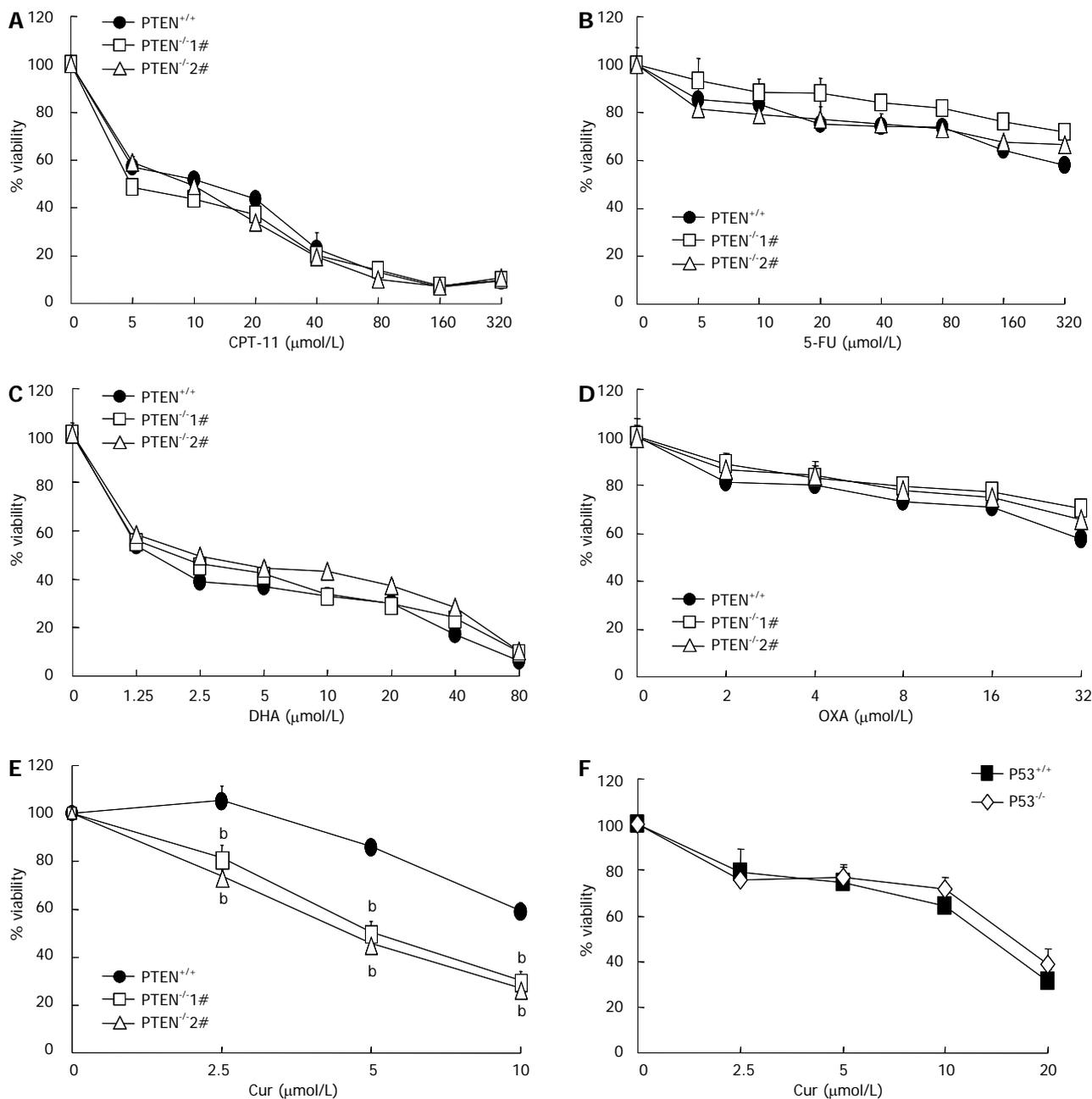


Figure 2 Curcumin shows enhanced cytotoxicity toward *PTEN*-deficient cells. A-E: Cytotoxic effects of irinotecan (CPT-11), 5-fluorouracil (5-FU), dihydroartemisinin (DHA), oxaliplatin (OXA) and curcumin (Cur) on cell viability of HCT116 phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*<sup>+/+</sup> and *PTEN*<sup>-</sup> cells were determined using the MTT assay; F: Cytotoxic effects of curcumin (Cur) on HCT116 *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> cells (<sup>b</sup>*P* < 0.01 vs control group).

ferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, United States). After blocking in 5% milk in TBST [10 mol/L Tris/HCl pH 7.4, 150 mol/L NaCl, and 0.1% (v/v) Tween-20], the membranes were incubated with primary antibodies, followed by secondary antibodies. Protein bands were visualized using an ECL system (Amersham Biosciences, United States).

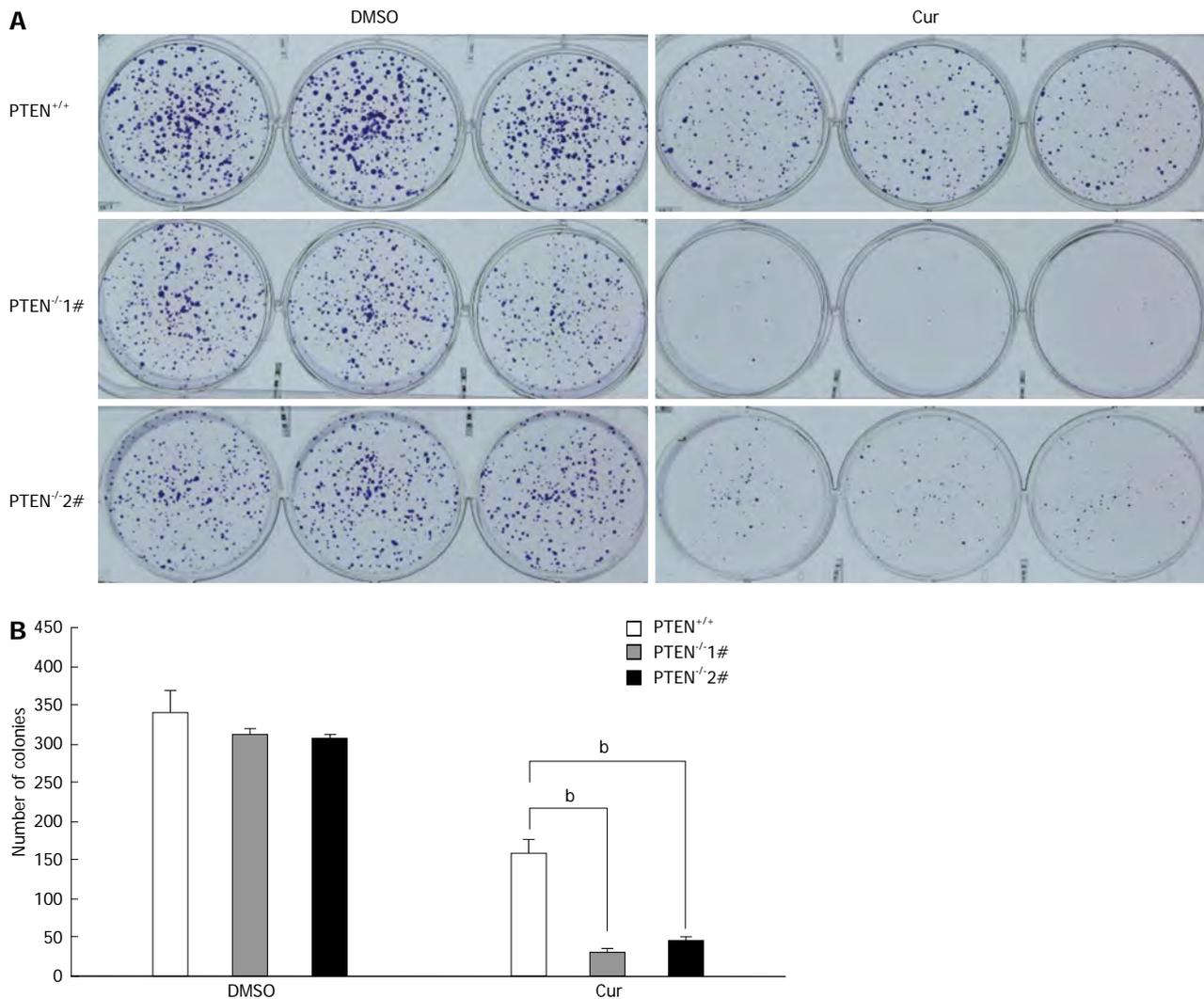
**Statistical analysis**

Data were summarized using means ± SD. Statistical comparisons were made using the Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

**RESULTS**

**Targeted deletion of *PTEN* in colorectal cancer cells**

Recombinant adeno-associated virus vectors were used to disrupt the endogenous *PTEN* gene in a near-diploid colon cancer cell line, HCT116, containing two wild-type alleles of *PTEN*. Targeting was directed to the exon 4 of *PTEN*, which resulted in a frame-shift mutation and thus a loss of *PTEN* expression. The detailed strategy is depicted in Figure 1A. The first allele of *PTEN* was disrupted by homologous recombination with the *PTEN*-targeting vector. The *PTEN*<sup>+/−</sup> clones were confirmed



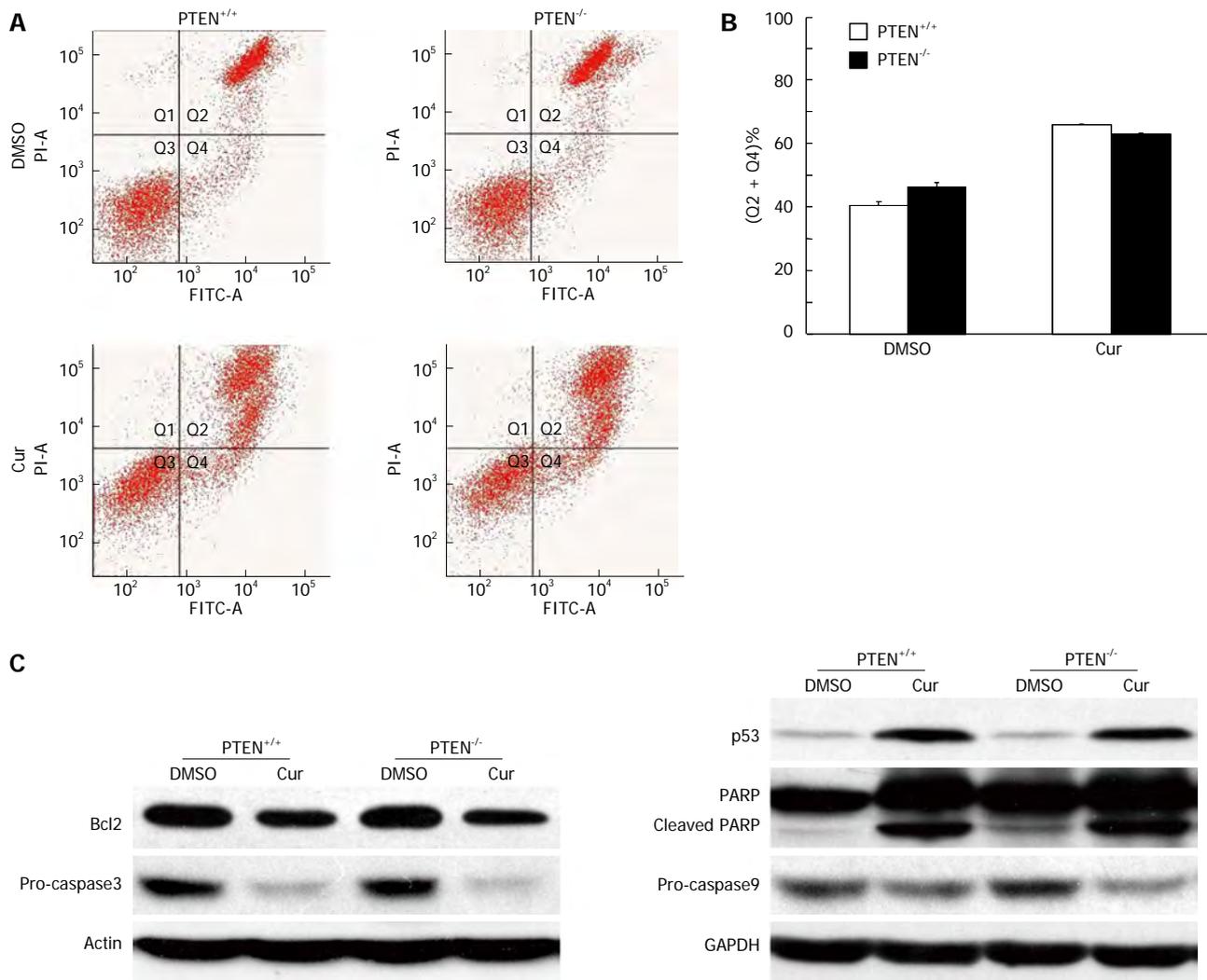
**Figure 3** Curcumin has an increased growth-inhibitory effect on *PTEN*-deficient cells in clonogenic assays. **A:** Photographs of clonogenic assay plates; **B:** Histograms showing clonogenic assay results (<sup>a</sup>*P* < 0.01 vs control group). Cur: Curcumin.

by PCR and transiently exposed to Cre recombinase, mediating the excision of the internal neomycin selection cassette flanked by loxP sites. A *PTEN*<sup>+/+</sup> clone was then used to generate *PTEN*<sup>-/-</sup> cells *via* a second round of recombination with the *PTEN*-targeting vector. The absence of *PTEN* mRNA in *PTEN*<sup>-/-</sup> clones was verified by PCR (Figure 1B). Western blotting analysis confirmed a decrease in *PTEN* levels in *PTEN*<sup>-/-</sup> cells and a complete loss of *PTEN* expression in *PTEN*<sup>-/-</sup> clones, which was accompanied by increased AKT phosphorylation (Figure 1C). Deletion of exon 4 of the *PTEN* gene was further verified by sequencing (Figure 1D). The *PTEN*<sup>-/-</sup> cells displayed a similar morphology to the parental *PTEN*<sup>+/+</sup> cell line (Figure 1E). The effect of *PTEN* deficiency on the viability of HCT116 cells was assessed using the MTT assay. However, no significant difference in cell growth characteristics was observed between *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells (Figure 1F).

#### Curcumin shows enhanced cytotoxicity toward *PTEN*-deficient cancer cells

*PTEN* acts as a tumor suppressor and its status is as-

sociated with sensitivity to chemotherapeutic agents<sup>[23,24]</sup>. Therefore, we investigated whether *PTEN* disruption affected the cytotoxicity of several clinical drugs and natural anti-cancer compounds. The *PTEN*-deficient cells and isogenic *PTEN* positive cells were exposed to increasing concentrations of anti-cancer compounds and their viability was determined. Disruption of the *PTEN* gene had no effect on the sensitivity of HCT116 cells to 5-FU, CPT-11, DHA, or OXA (Figure 2A-D). Surprisingly, *PTEN*-deficient cells were more sensitive to curcumin as compared with the parental *PTEN*<sup>+/+</sup> cells. The IC<sub>50</sub> value of curcumin for HCT116 *PTEN*<sup>-/-</sup> cells was approximately 2-fold lower than that for *PTEN*<sup>+/+</sup> cells (Figure 2E). Next, we determined whether curcumin also showed increased cytotoxicity toward HCT116 cells deficient in p53. However, we found that disruption of p53 had no effect on the sensitivity of HCT116 cells to curcumin (Figure 2F). The fact that *PTEN* deficiency resulted in increased sensitivity of HCT116 cells to curcumin was further confirmed using a clonogenic assay. Following curcumin exposure, a significantly smaller number of colonies was observed with HCT116 *PTEN*<sup>-/-</sup>



**Figure 4** *PTEN* deficiency does not enhance curcumin-induced apoptosis. **A**: Images showing flow cytometric analysis of apoptosis. Apoptosis was analyzed by flow cytometry with the Annexin V-FITC kit; **B**: Histograms showing apoptosis assay results. Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) deletion did not result in increased apoptosis following curcumin treatment; **C**: Western blotting analysis. Cell lysates were separated on SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with indicated antibodies. Cur: Curcumin; PI: Propidium iodide; FITC: Fluorescein isothiocyanate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

cells than with *PTEN*<sup>+/+</sup> cells (Figure 3). Together, these results suggest that curcumin exhibits enhanced cytotoxicity toward HCT116 cells deficient in *PTEN*.

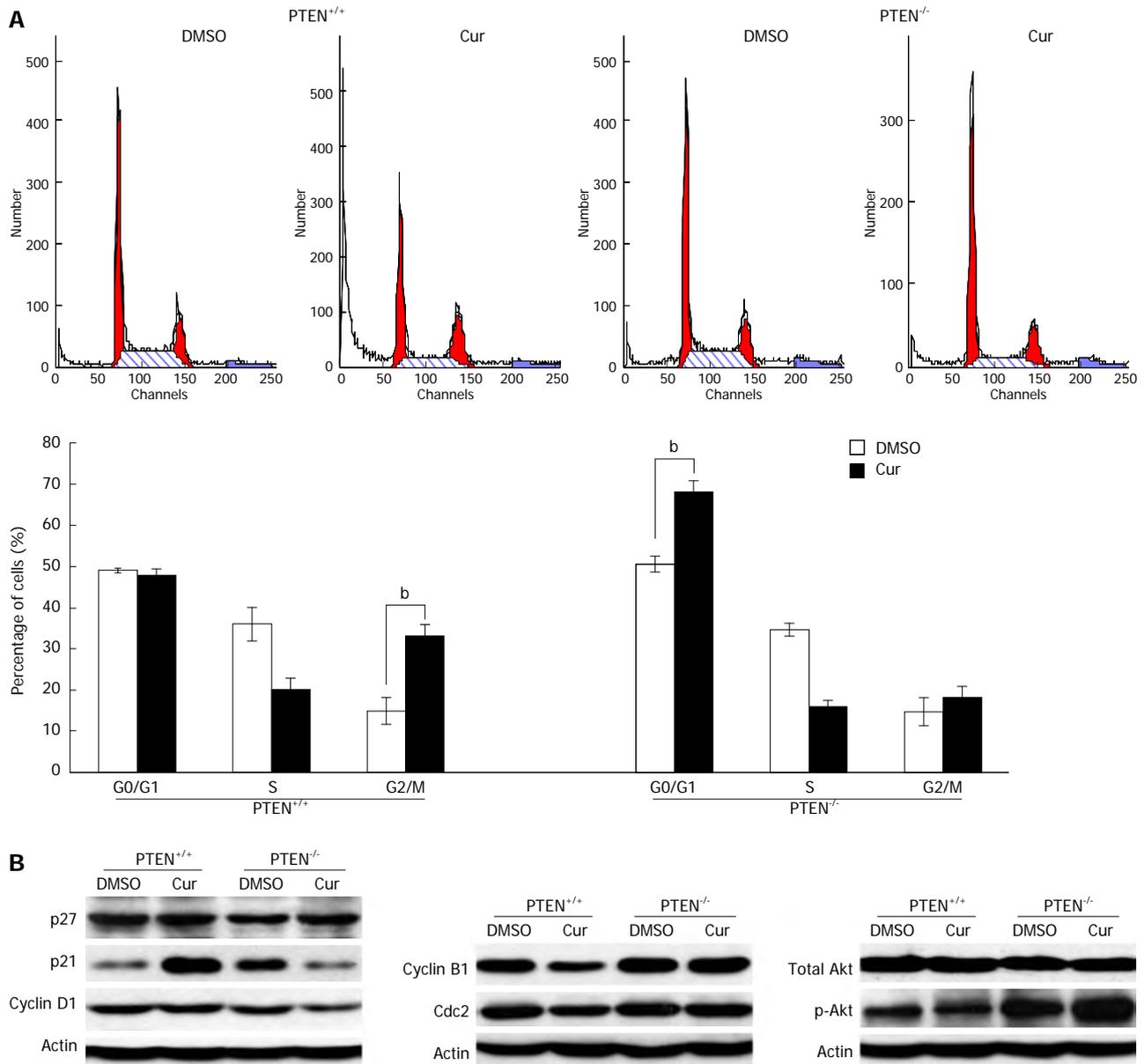
***PTEN* deficiency does not increase curcumin-induced apoptosis**

Curcumin treatment induces cell apoptosis<sup>[25]</sup>. To understand the molecular mechanism underlying the increased cytotoxicity of curcumin toward *PTEN*-deficient CRC cells, we tested whether *PTEN* loss resulted in increased curcumin-mediated apoptosis. Using flow cytometric analysis with FITC-labeled annexin V and propidium iodide staining, we observed significantly increased apoptosis in both HCT116 *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells after curcumin exposure. However, the apoptotic index was not affected by disruption of the *PTEN* gene, being similar in *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells (Figure 4A and B). In accordance with these results, the expression patterns of apoptosis-related proteins, such as Bcl-2, procaspase 3 and 9, p53 and PARP were similar in both cell lines fol-

lowing curcumin exposure (Figure 4C). Therefore, these data suggest that the enhanced cytotoxicity of curcumin toward *PTEN*<sup>-/-</sup> colon cancer cells is not due to an increase in curcumin-induced apoptosis.

***PTEN* deficiency results in altered curcumin-induced cell cycle arrest**

Next, we tested whether the enhanced cytotoxicity of curcumin to *PTEN*-deficient cells was caused by altered cell cycle progression. Consistent with a previous report<sup>[26]</sup>, we found that curcumin exposure led to a marked G2/M phase cell cycle arrest in HCT116 cells with wild-type *PTEN* (Figure 5A). Surprisingly, in *PTEN*<sup>-/-</sup> cells, curcumin treatment resulted in a significant accumulation of cells in G0/G1 phase, characteristic of a G0/G1 phase arrest (Figure 5A). To explore the mechanism underlying the altered cell cycle arrest pattern, we investigated the expression of proteins related to AKT signaling and the cell cycle. Curcumin exposure marginally reduced AKT phosphorylation and significantly induced p21 ex-



**Figure 5** Loss of *PTEN* alters curcumin-mediated cell cycle arrest. **A:** Cell cycle distributions of phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*<sup>+/+</sup> (left) and *PTEN*<sup>-/-</sup> (right) cell samples were analyzed by flow cytometry (<sup>b</sup>*P* < 0.01 vs control group); **B:** Western blotting analysis. Whole cell lysate isolated from *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and incubated with the indicated antibodies. Cur: Curcumin.

pression in HCT116 cells harboring wild-type *PTEN*. Interestingly, the opposite results were observed in HCT116 *PTEN*<sup>-/-</sup> cells, which showed a significant induction of AKT phosphorylation. Curcumin exposure further elevated this increase in AKT phosphorylation, which was accompanied by a significant reduction in p21 expression (Figure 5B). In agreement with the differential expression of p21 in curcumin-treated *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells, we observed a down-regulation of Cyclin B1 and Cdc2 in *PTEN*<sup>+/+</sup> cells, whereas decreased expression of Cyclin D1 was observed in *PTEN*<sup>-/-</sup> cells (Figure 5B), consistent with their respective patterns of cell cycle arrest. p27 is another known regulator of G0/G1 phase; however, we observed no difference in its expression levels between the *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells following curcumin

exposure (Figure 5B). Together, these data suggest that the enhanced cytotoxicity of curcumin caused by *PTEN* deficiency might be due to an altered pattern of cell cycle arrest that is related to the *PTEN*/AKT/p21 pathway.

## DISCUSSION

*PTEN* is a tumor suppressor gene that is frequently mutated in human colorectal cancers. *PTEN* functions as a lipid phosphatase that negatively regulates PI3K/AKT signaling, which is critical for cell proliferation, apoptosis, and cell cycle progression<sup>[27]</sup>. *PTEN* mutations result in aberrant activation of the AKT pathway, facilitating cancer progression and causing drug resistance in CRC<sup>[28,29]</sup>. Therefore, chemotherapeutic agents, such as natural

products and synthetic compounds, that can enhance therapeutic efficacy for CRC with PTEN mutations, are urgently needed. In the present study, we found that curcumin showed enhanced cytotoxicity toward CRC cells deficient in PTEN. This intriguing finding indicates that curcumin might be applied alone or in combination with other chemotherapeutic agents for the therapy of PTEN-mutant cancers.

Curcumin is a natural pigment extracted from the roots of the turmeric plant (*Curcuma longa*). In addition to its anti-oxidative and anti-inflammatory properties, curcumin inhibits cell proliferation in a number of cancer cell lines, including those derived from colon, breast, lung and bladder cancers<sup>[30-33]</sup>. Moreover, curcumin inhibits tumorigenesis *in vivo*. It has been shown that curcumin effectively prevents tumor implantation and growth in mice, and suppresses the development of bladder cancer in a rat model<sup>[34,35]</sup>. Mechanistic studies demonstrated that curcumin targets a number of molecules, including apoptosis-related proteins such as Bcl-2, caspase 3 and 9, as well as cell cycle regulators<sup>[36,37]</sup>. In this study, we report for the first time that the enhanced cytotoxicity of curcumin toward PTEN-deficient cells is not due to an increase in apoptosis, but rather to altered cell cycle arrest patterns, from the G2/M phase arrest seen in *PTEN*<sup>+/+</sup> cells to a G0/G1 phase arrest in *PTEN*<sup>-/-</sup> cells.

The *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells used in this study differ only in their PTEN status. Since PTEN is an upstream regulator of AKT signaling, the differential effects of curcumin on cells with and without PTEN might be associated with altered AKT signaling. Consistent with this hypothesis, we observed differences in curcumin-induced AKT phosphorylation in *PTEN*<sup>+/+</sup> and *PTEN*<sup>-/-</sup> cells. In contrast to the slight decrease observed in p-AKT in *PTEN*<sup>+/+</sup> cells, p-AKT was significantly increased in *PTEN*<sup>-/-</sup> cells after curcumin exposure. p21 is a well-known downstream effector of AKT signaling. p-AKT can phosphorylate p21 and restrict it to the cytoplasm for degradation<sup>[38]</sup>. In accordance with this, p21 expression was significantly increased in *PTEN*<sup>+/+</sup> cells, but markedly decreased in *PTEN*<sup>-/-</sup> cells following curcumin exposure. Consequently, the increased expression of p21 led to a down-regulation of Cyclin B1 and Cdc2 and thus a G2/M phase arrest in *PTEN*<sup>+/+</sup> cells, which is consistent with a previous study<sup>[39]</sup>. Despite its function as a negative regulator of the cell cycle, p21 can also positively regulate cell cycle progression by serving as an assembly factor for the Cyclin D/Cdk4 complex and facilitating the transition from G1 phase to S phase<sup>[40-42]</sup>. Consistent with this role, we observed a decrease in p21 expression, accompanied by a reduced level of Cyclin D1 and G0/G1 arrest in *PTEN*<sup>-/-</sup> cells after curcumin exposure. Based on our findings, we speculate that PTEN deficiency alters AKT signaling and thus the expression of p21 induced by curcumin, and that this alteration results in an increased G0/G1 phase arrest, which may account for the enhanced curcumin sensitivity of PTEN-deficient cells. Our results suggest that p21 is a potential regulator in the enhanced cytotoxicity of curcumin toward PTEN-

deficient cells; however, the detailed mechanism remains to be elucidated.

In conclusion, we have shown that curcumin exhibits increased cytotoxicity toward PTEN-deficient cancer cells, and the underlying mechanism might involve cell cycle arrest alterations that are associated with the PTEN/AKT/p21 pathway. These findings also suggest that curcumin might potentially contribute to the therapy of PTEN-mutated cancers.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Resistance to chemotherapeutic drugs remains a major obstacle to the effective treatment of cancers. Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is one of the most frequently mutated tumor suppressor genes in human cancers. Accordingly, mutational inactivation of PTEN leads to cancer progression and chemotherapy resistance. Therefore, chemotherapeutic agents with greater efficacy for cancer with PTEN mutations are urgently required.

### Research frontiers

Curcumin is a major yellow-colored dietary pigment from *Curcuma longa*. Curcumin has demonstrated anticancer properties in various cancers, including colon cancer. This study analyzes the possible effects of curcumin on colorectal cancer cells with PTEN deficiency.

### Innovations and breakthroughs

By gene targeting *PTEN* in HCT116 cells we created PTEN null HCT116 cells, which are isogenic to the PTEN positive HCT116 parental cell line. The authors found that PTEN null cells exhibited greater sensitivity to curcumin than PTEN positive cells. The authors further established that this difference was not due to an increase in apoptosis, but the result of altered cell cycle arrest induced by curcumin in PTEN null cells.

### Applications

Curcumin is a potential chemotherapeutic agent for PTEN mutant cancers, and could be used in individualized cancer therapy. The PTEN-deficient cell line might be a useful tool for screening chemotherapeutic agents against PTEN-mutant cancers.

### Terminology

The PI3K/AktKT pathway is an intracellular signaling pathway important for cell growth, survival, cell cycle progression, and apoptosis.

### Peer review

This is the first report of the effects of PTEN deficiency on the cytotoxicity of curcumin for colorectal cancer cells. The authors found that PTEN deficiency affects the cytotoxicity of curcumin on HCT116 cells. Upon further investigation they found that this effect is related to increased G0/G1 arrest but not to increased apoptosis. The manuscript is consistent and well structured.

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## Acute arterial mesenteric ischemia and reperfusion: Macroscopic and MRI findings, preliminary report

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### Abstract

**AIM:** To explore the physiopathology and magnetic resonance imaging (MRI) findings in an animal model of acute arterial mesenteric ischemia (AAMI) with and without reperfusion.

**METHODS:** In this study, 8 adult Sprague-Dawley rats

underwent superior mesenteric artery (SMA) ligation and were then randomly divided in two groups of 4. In group I, the ischemia was maintained for 8 h. In group II, 1-h after SMA occlusion, the ligation was removed by cutting the thread fixed on the back of the animal, and reperfusion was monitored for 8 h. MRI was performed using a 7-T system.

**RESULTS:** We found that, in the case of AAMI without reperfusion, spastic reflex ileus, hypotonic reflex ileus, free abdominal fluid and bowel wall thinning are present from the second hour, and bowel wall hyperintensity in T2-W sequences are present from the fourth hour. The reperfusion model shows the presence of early bowel wall hyperintensity in T2-W sequences after 1 h and bowel wall thickening from the second hour.

**CONCLUSION:** Our study has shown that MRI can assess pathological changes that occur in the small bowel and distinguish between the presence and absence of reperfusion after induced acute arterial ischemia.

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**Key words:** Acute arterial mesenteric ischemia; Reperfusion; Magnetic resonance imaging; Animal model; Superior mesenteric artery; Bowel ischemia

**Core tip:** Diagnosis of acute arterial mesenteric ischemia depends on early detection and findings with regarding the presence or absence of reperfusion events. Distinguishing between these different conditions is crucial to improving outcome for the patient and represents a challenge for radiologists. The results of this preliminary study in an animal model provide for a time-based definition of the radiological findings in ischemia and reperfusion, showing that magnetic resonance imaging can adequately assess the different pathological changes that occur in acute arterial mes-

## enteric ischemia with or without reperfusion.

Saba L, Berritto D, Iacobellis F, Scaglione M, Castaldo S, Cozzolino S, Mazzei MA, Di Mizio V, Grassi R. Acute arterial mesenteric ischemia and reperfusion: Macroscopic and MRI findings, preliminary report. *World J Gastroenterol* 2013; 19(40): 6825-6833 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6825.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6825>

## INTRODUCTION

Mesenteric ischemia can have various causes<sup>[1]</sup>. One of the most threatening conditions is acute arterial mesenteric ischemia (AAMI), which is characterized by the acute occlusion of the arteries feeding the small bowel<sup>[2]</sup>. AAMI is considered a vascular emergency with a mortality rate of up to 60%-80%<sup>[3]</sup>.

Mortality of patients with AAMI most likely remains high because ischemic changes of the abdominal organs are most frequently detected and diagnosed at a late stage when therapy is ineffective<sup>[4,5]</sup>. Multi-detector computed tomography angiography is currently considered the gold-standard for imaging mesenteric and colonic ischemia<sup>[6,7]</sup>. However, recent papers have shown that magnetic resonance imaging (MRI) has significant potential for detecting subtle changes that may affect the intestinal wall during the ischemic process<sup>[8]</sup>.

Recently, some researchers<sup>[9]</sup> assessed the evolution of ischemic lesions using 7 Tesla-magnetic resonance imaging (7T-MRI) in an animal model of acute colonic ischemia. This work demonstrated that MRI can be used as a reliable diagnostic and grading technique in acute ischemic colitis, which would allow for the early identification of pathology.

The purpose of this study was to explore physiopathology and MRI findings of mesenteric ischemia in an animal model, with and without reperfusion, to define semiological changes of mesenteric ischemia from both a macroscopic and MRI point-of-view.

## MATERIALS AND METHODS

### *Animal preparation*

All procedures performed on animals were approved by the Animal Care and Use Committee of the Biotechnology Center of Cardarelli Hospital. Eight adult male Sprague-Dawley rats (250-340 g; Harlan, United States) were randomly divided in two groups of 4 rats each (group I and group II). The rats were maintained on a 12/12 h light/dark cycle and allowed free access to food and water. They were anesthetized with ketamine hydrochloride 100 mg/kg IM (CU ChemieUetikon GmbH, Lahr, Germany) and Domitor 0.25 mg/kg IM (Medethomidine hydrochloride, Pfizer, NY, United States) injections. Dolorex 0.1 mg/kg *sc* (Butarphanol, Intervet,

Boxmeer, The Netherlands) was used immediately before the procedure to ensure intra-operative analgesia. Further injections of these drugs were provided throughout the operation to maintain a sufficient state of anesthesia. Each rat was allowed to breathe spontaneously. Body temperature was monitored with a rectal probe and maintained at  $37.0 \pm 0.5$  °C with a heating blanket regulated by a homeothermic blanket control unit (Harvard Apparatus Limited, Edenbridge Kent, United Kingdom). After drug injection, rats were prepared for surgery via abdominal depilation. The area was then washed with povidone iodine and alcohol.

### *Surgical procedures*

Surgery and drug administration were performed by a board-certified veterinarian with 5 years of experience in microsurgical and vascular techniques (Scaglione M). In all rats, acute SMA occlusion was induced using a two-step surgical procedure (Figure 1). After midline laparotomy, the bowel was exposed in the abdominal cavity and displaced to the left, allowing identification of the SMA. The bowel and the mesentery were drawn out of the abdominal cavity and a snapshot of exposed organs was taken (Nikon Coolpix S210, 8.0 Megapixels resolution, ISO 2000, Japan) as a basal image.

A 3/0 silk thread was used to wind a loop around the SMA at the origin, not yet tightening the loop. The tips of the thread were brought into a silicon pipe and, through it, carried out to the back of the animal, between its shoulders. The thread tips were attached to the pipe using medical plaster. Muscles and skin were closed in two layers using a 2/0 Vicryl thread. 10 mg/kg of Baytril 10% (Enrofloxacin 2.5%, Bayer AG, Leverkusen, Germany) was topically applied to the wounds to prevent infections. The animals were returned to their cages after awakening, and water and food were provided *ad libitum*. Three days after surgery, the rats underwent a second anesthetic treatment according to the same drug protocol, and acute mesenteric ischemia was induced by pulling the threads out of the pipe. In this way, the loop around the SMA was squeezed, and the arterial inflow through this artery was stopped. In group I rats, the ischemia was maintained for 8 h and during this time all animals were monitored at predetermined time points. In group II rats, the ligation was removed 1 h after SMA occlusion by cutting the thread fixed on the back of the animal; the following reperfusion was monitored for 8 h.

### *7T-magnetic resonance imaging protocol*

All animals underwent MRI of the abdomen using a 7-T micro-MRI scanner (BioSpec 70/16US, Bruker Medical Systems, Ettlingen, Germany). Two abdominal radiologists (Saba L and Grassi R), with 10 and 15 years of experience, respectively, assessed all 7T MR images by consensus. In all the rats, just before pulling the threads and occluding the vessel, a localizer scan along the three orthogonal planes [repetition time (TR) = 6.0 ms, echo time (TE) = 100.0 ms, field of view (FOV) = 8, Averages

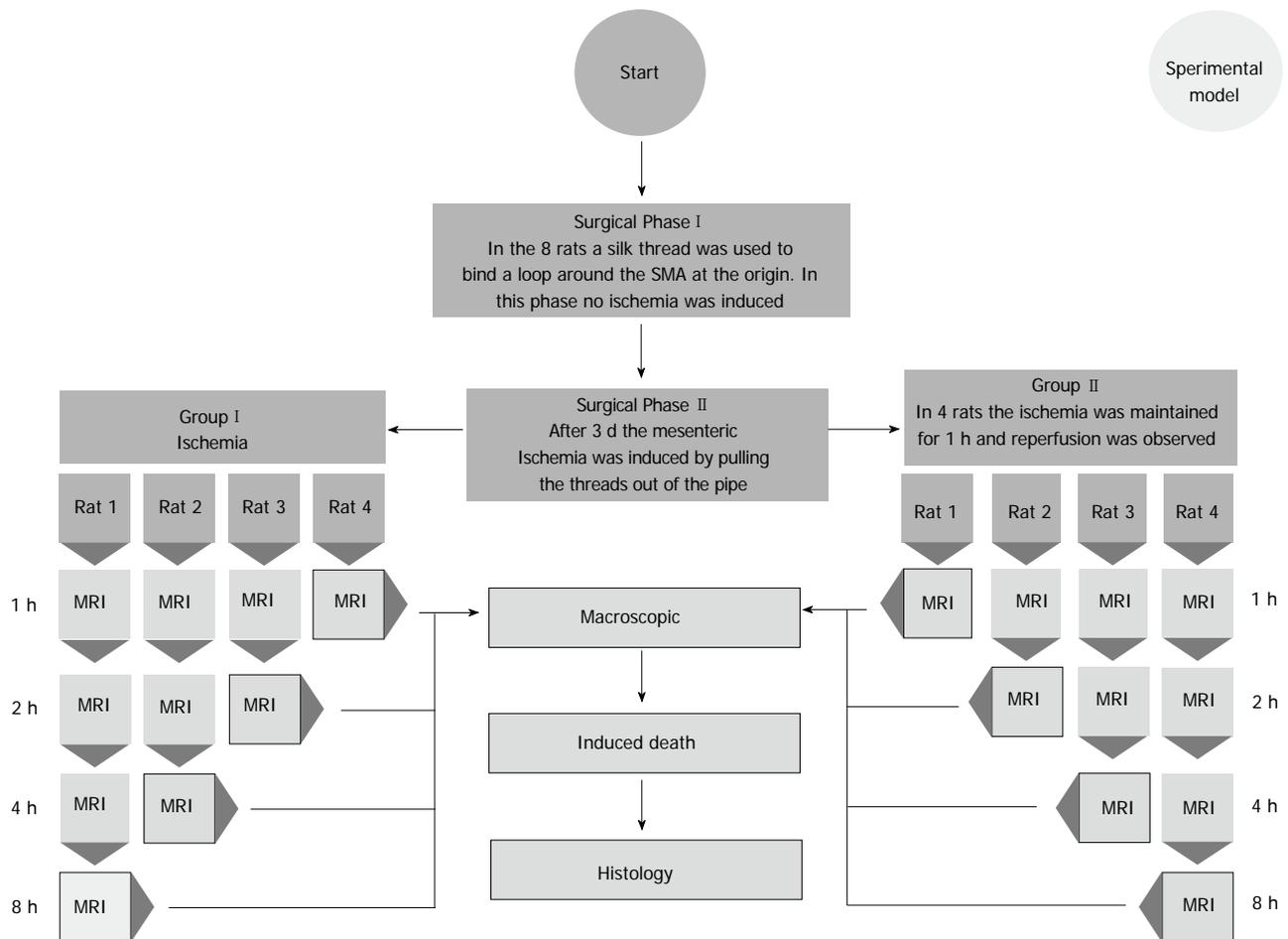


Figure 1 Flow chart. Flow chart shows our procedure to study the 2 different groups of rats. MRI: Magnetic resonance imaging; SMA: Superior mesenteric artery.

= 1, Flip Angle 30 deg, Matrix = 128, Slice thickness = 2.00 mm, Scan time = 12 s 800 ms] and a T2-weighted (T2-W) TurboRare sequence (TR = 4428.7 ms, TE = 36 ms; 180° flip angle; 38 slices; slice thickness 1 mm, interslice distance 1 mm, field of view, 6 cm; acquisition matrix, 256 × 256, scan time = 7 min 5 s 156 ms) were performed to serve as baseline images. Before and after the SMA occlusion, to insure the complete lack of flow in the vessels, a FLASH TOF 2D sequence was performed (TR = 12 ms, TE = 3.5 ms; 90° flip angle; 180 slices; slice thickness 0.4 mm, interslice distance 0.25 mm, field of view, 6 cm; acquisition matrix, 256 × 256, averages = 7, acquisition time 48 min 23 s 40 ms). Maximum intensity projection (MIP) images were reconstructed for each series of images to evaluate SMA occlusion.

For those rats in which lack of flow in the SMA after occlusion was confirmed, additional T2-W TurboRare sequences were performed at predetermined time-points to identify and characterize signs of bowel necrosis. For group II rats, a second FLASH TOF 2D sequence (TR = 12 ms, TE = 3.5 ms; 90° flip angle; 180 slices; slice thickness 0.4 mm, interslice distance 0.25 mm, field of view, 6 cm; acquisition matrix, 256 × 256, averages = 7, acquisition time 48 min 23 s 40 ms) after reperfusion was performed to verify the bloodflow through the SMA.

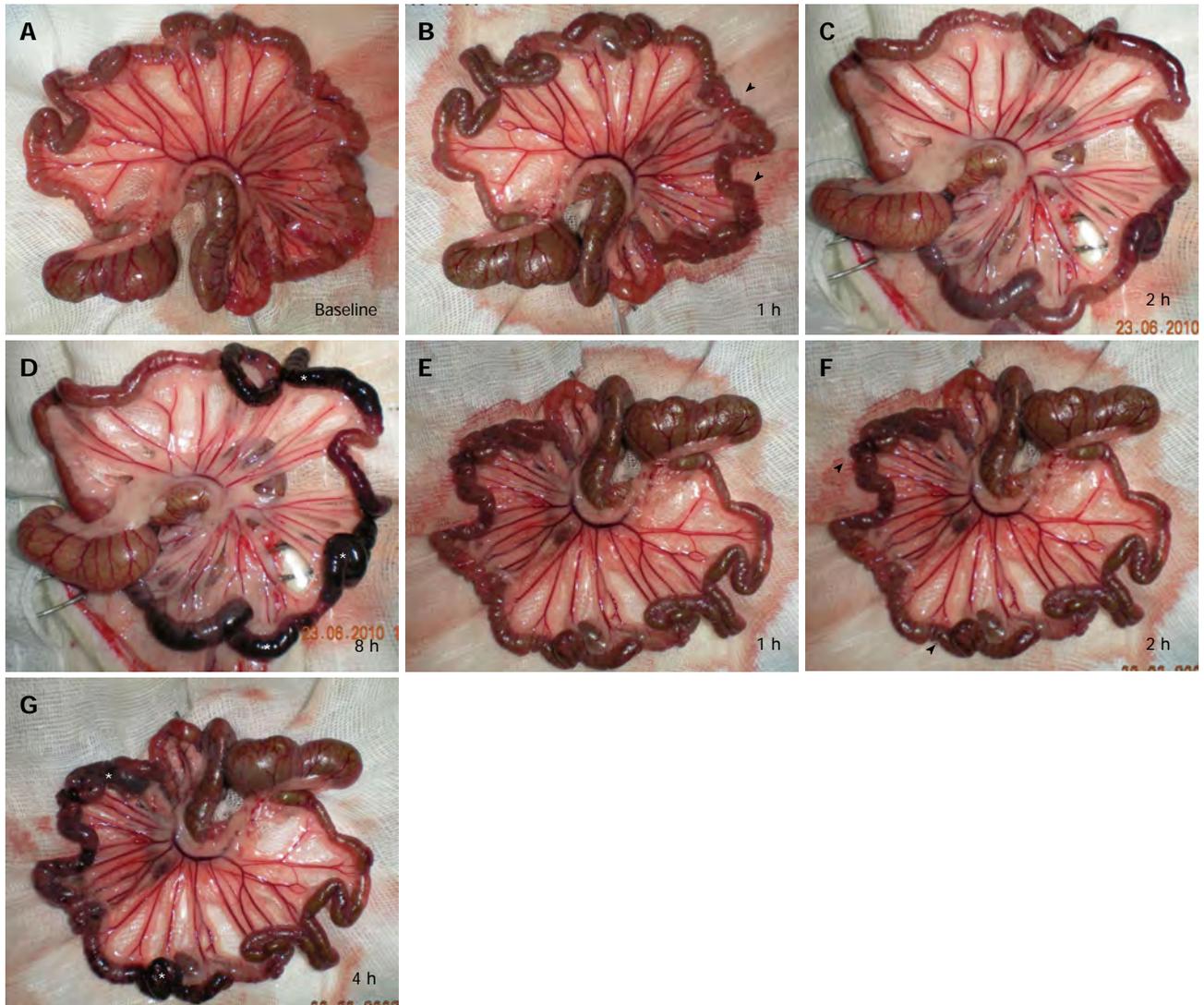
At each time-point in each rat, after opening the pre-existing midline laparotomy, the bowel and the mesentery were drawn out of the abdominal cavity, and a picture of the exposed organs was taken (Nikon Coolpix S210, 8.0 Megapixels, ISO 2000, Japan). The rat was then euthanized by an intrapulmonary injection of Tanax 0.5 mL (Enbutramide + Mebenzonium, Iodide + Tetracaine, Intervet/Shering-Plough Animal Health, Boxmeer; The Netherlands).

MR images were assessed for the following parameters: (1) presence of free fluid in the abdomen; (2) gas filled dilated loops, known as hypotonic reflex ileus (HRI); (3) gas-fluid mixed stasis dilated loops (paralytic ileus, PI); (4) bowel wall thinning or thickening; (5) wall signal intensity on T2-W sequences; (6) mesenteric vessels; (7) wall pneumatosis; and (8) presence of free gas in the abdomen.

## RESULTS

### Macroscopic analysis

Immediately before the SMA ligation the bowel loops presented an average diameter of 1.5 mm (measured with a gauge), uniform serosa and rose-colored mesentery in all segments (Figure 2A). In all rats, 1 h after ischemia



**Figure 2 Macroscopic appearance.** A-D: Group I ; at baseline evaluation, the bowel loops presented an average diameter of 1.5 mm, uniform serosa and rose-colored mesentery in all segments (A). Pale mesentery and spasm of the jejunal loops (arrowheads) were evident 1 h after acute superior mesenteric artery occlusion (B). Spastic reflex ileus was replaced by definite hypotonic reflex ileus 2 h after acute ischemia (C). Eight hours from acute superior mesenteric artery occlusion, a chromatic change to charcoal black was extended to a large ileal segment (white stars) (D); E-G: Group II ; vascular congestion of mesenteric vessels was evident 1 h after reperfusion (E). After 2 h from reperfusion, damaged areas underwent chromatic change from pink to dark red (arrowheads) (F), and became charcoal black after 4 h (stars) (G).

(Figure 2B), pale mesentery with thinning of mesenteric vessels and a spasm of the jejunal loops were evident, compared with the baseline assessment.

In group I rats, 2 h after acute SMA occlusion the spasm was replaced by definite HRI (Figure 2C). The ileal loops underwent the same pathological evolution, but the spasm was observed approximately 2 h after acute SMA occlusion, with transition to definite HRI after approximately 4 h. At this stage, a chromatic change from pink to dark red was observed in the loops of the middle portion of the ileum, then became charcoal black in the rat observed for 8 h, spreading to a cover larger ileal segment - both proximal and distal (Figure 2D). In group II rats, vascular congestion of mesenteric vessels was evident 1 hour after reperfusion (Figure 2E). After 2 h of reperfusion, damaged areas underwent chromatic change from pink to dark red, then became charcoal black at 4 h

and growing more apparent in subsequent hours (Figure 2F and G).

### 7T MRI

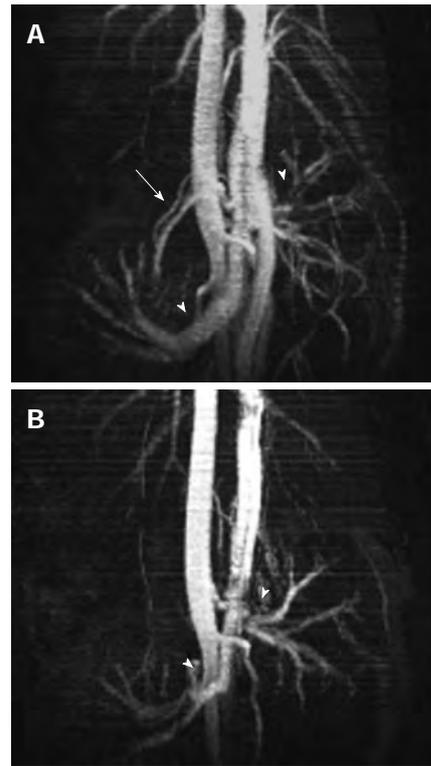
In all cases 7-T micro-MRI identified findings of intestinal ischemia. Immediately before inducing acute SMA obstruction, 3 d after laparotomy, no free gas within the abdominal cavity or signs of visceral or mesentery irritation were found. Compared with the baseline acquisition, the MR angiography sequence at 5 min showed that flow in the SMA had stopped (Figure 3). In group I (Table 1), 1 h after SMA ligation, T2-W sequences showed minimal change - a thin collection of fluid between the loops - compared with control scans (Figure 4A and B).

Dilation of numerous bowel loops (HRI) (mean diameter 4 mm, compared with 2.5 mm in baseline acquisition) and reduced wall thickness (average 0.5 mm com-

**Table 1** Magnetic resonance imaging findings

	1-h	2-h	4-h	8-h
<b>Group I : Magnetic resonance imaging findings</b>				
Rat 1 Free fluid	1	1	1	1
Free gas	0	0	0	0
HRI	0	1	1	1
PI	0	0	1	1
Bowel wall thinning	0	1	1	1
Bowel wall thickening	0	0	0	0
Wall signal (T2-W sequences)	0	0	1	1
Wall pneumatosis	0	0	0	0
Rat 2 Free fluid	1	1	1	Rat dead
Free gas	0	0	0	
HRI	0	1	1	
PI	0	0	1	
Bowel wall thinning	0	1	1	
Bowel wall thickening	0	0	0	
Wall signal (T2-W sequences)	0	0	1	
Wall pneumatosis	0	0	0	
Rat 3 Free fluid	1	1		Rat dead
Free gas	0	0		
HRI	0	1		
PI	0	0		
Bowel wall thinning	0	1		
Bowel wall thickening	0	0		
Wall signal (T2-W sequences)	0	0		
Wall pneumatosis	0	0		
Rat 4 Free fluid	1			Rat dead
Free gas	0			
HRI	0			
PI	0			
Bowel wall thinning	0			
Bowel wall thickening	0			
Wall signal (T2-W sequences)	0			
Wall pneumatosis	0			
<b>Group II : Magnetic resonance imaging findings</b>				
Rat 5 Free fluid	1	1	1	1
Free gas	0	0	0	0
HRI	0	1	1	1
PI	0	0	1	1
Bowel wall thinning	0	0	0	0
Bowel wall thickening	0	1	1	1
Wall signal (T2-W sequences)	1	1	1	1
Wall pneumatosis	0	0	0	0
Rat 6 Free fluid	1	1	1	Rat dead
Free gas	0	0	0	
HRI	0	1	1	
PI	0	0	1	
Bowel wall thinning	0	0	0	
Bowel wall thickening	0	1	1	
Wall signal (T2-W sequences)	1	1	1	
Wall pneumatosis	0	0	0	
Rat 7 Free fluid	1	1		Rat dead
Free gas	0	0		
HRI	0	1		
PI	0	0		
Bowel wall thinning	0	0		
Bowel wall thickening	0	1		
Wall signal (T2-W sequences)	1	1		
Wall pneumatosis	0	0		
Rat 8 Free fluid	1			Rat dead
Free gas	0			
HRI	0			
PI	0			
Bowel wall thinning	0			
Bowel wall thickening	0			
Wall signal (T2-W sequences)	1			
Wall pneumatosis	0			

HRI: Hypotonic reflex ileus; PI: Paralytic ileus.

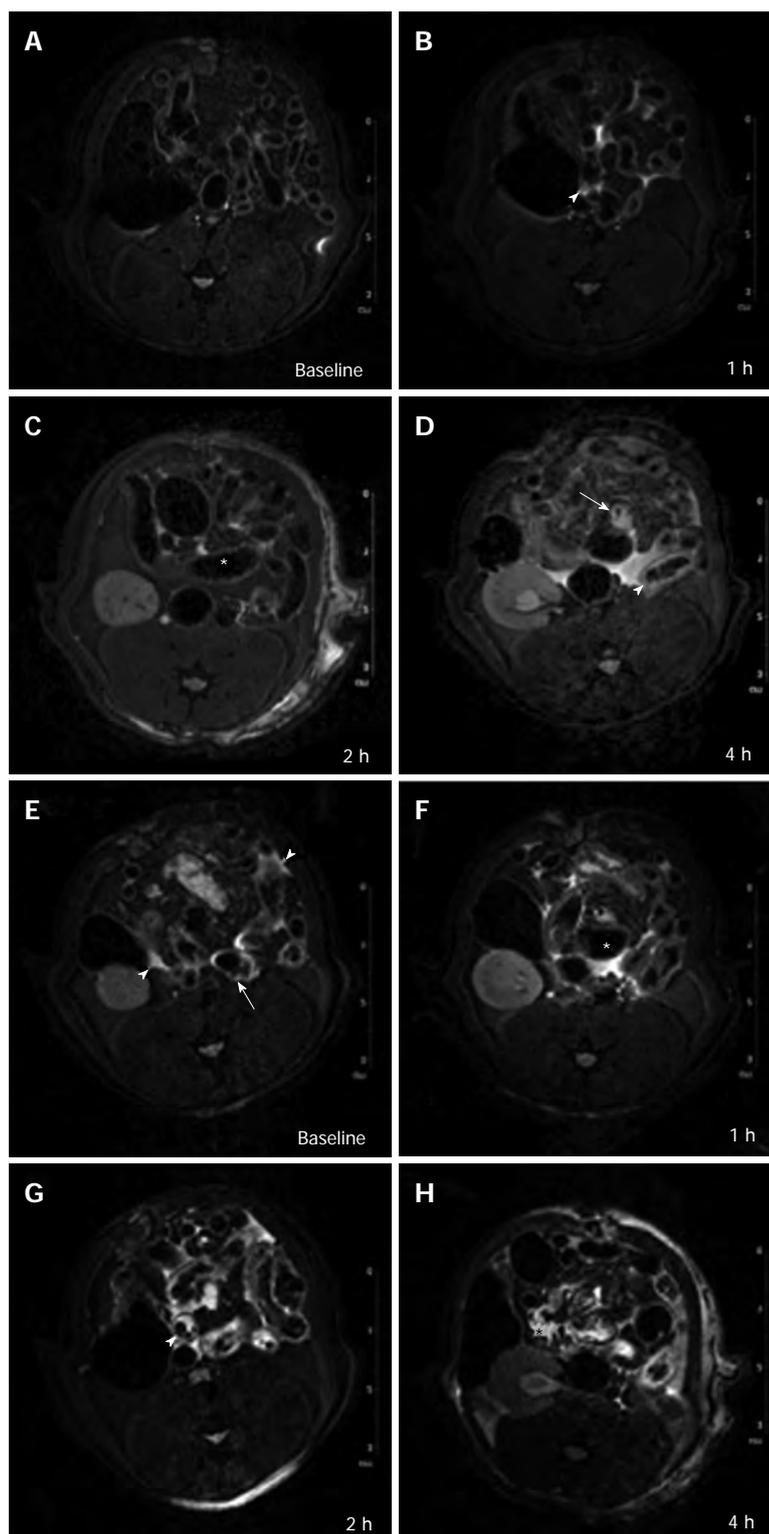
**Figure 3** Magnetic resonance imaging angiography. Maximum intensity projection images through origins of renal arteries (arrowheads) and acute superior mesenteric artery (SMA) (arrow) before (A), and after SMA occlusion (B) (FLASH 2D-TOF), no flow is observed in SMA (B).

pared with 1 mm in baseline acquisition) were already apparent at approximately 2 h of ischemia (Figure 4C). HRI was followed by PI (gas-liquid stasis) that was clearly evident 4 h after SMA ligation. A hyper-intense signal in the bowel wall was evident after 4 h in some loops (Figure 4D).

In group II (Table 1), an early hyper-intense signal of the bowel wall in some loops and a thin layer of peritoneal fluid were detected 1 h after reperfusion (Figure 4E). After 2 h, a small amount of peritoneal free fluid, bowel wall thickening (average thickness of 1.5 mm) and hyperintensity of the intestinal wall (Figure 4F) were observed. HRI in some loops and additional free fluid were related to previous findings. At 4 h, a PI (average diameter of intestinal lumen of 4.5 mm) with a larger amount of peritoneal fluid, a mild mesenteric engorgement and a hyper-intense signal of the intestinal wall became evident in many loops (Figure 4G and H). By comparing the different MRI signal characteristics of the rats that underwent the reperfusion versus those that did not undergo the reperfusion (Table 2), we found differences between the appearances of bowel wall thickening and wall signal (in T2-W).

## DISCUSSION

The morbidity and mortality of mesenteric ischemia are quite high<sup>[10]</sup>; some authors assert that this high mortality is associated with the low sensitivity of diagnostic tools, which may hinder a correct diagnosis in the early



**Figure 4** 7 Tesla-magnetic resonance imaging appearance. A-D: Group I ; at baseline evaluation no free gas within the abdominal cavity or signs of visceral or mesentery irritation were found on T2-W sequences (A). One hour after acute superior mesenteric artery (SMA) ligation, T2-W sequences showed a thin collection of fluid between the loops (arrowhead) (B). After 2 h dilation of numerous bowel loops [(hypotonic reflex ileus (HRI)] with reduced wall thickness were evident (star) (C). Four hours from SMA ligation HRI was followed by paralytic ileus (PI) (gas-liquid stasis) (arrow); also bowel wall hyper-intense signal was evident in some loops (arrowhead) (D). E-H: Group II ; An early hyper-intense signal of bowel wall in some loops (arrow) and a thin layer of peritoneal fluid (arrowheads) were detected 1 h after reperfusion (E). After 2 h, a small amount of peritoneal free fluid, bowel wall thickening, hyper-intense signal of some loops and HRI (star) were observed (F). At 4 h, a paralytic ileus (arrowhead) (G) with a larger amount of peritoneal fluid appeared and a mild mesenteric engorgement (star) became evident beyond a hyper-intense signal of intestinal wall in many loops (H).

phase of ischemia<sup>[11,12]</sup>. Early identification of mesenteric ischemia is critical to plan an appropriate therapeutic approach. Moreover, different characteristics of the bowel corresponding to different pathogenesis are not clearly defined in imaging studies, adding further difficulties to obtaining an early and precise diagnosis<sup>[13]</sup>.

We defined the progress of 2 different mechanisms of mesenteric ischemia - the acute occlusion of SMA and the acute occlusion of SMA followed by reperfusion

- and investigated MRI findings in a time course. Most studies about ischemia/reperfusion reported in literature<sup>[14]</sup> analyze histological or biochemical alteration. In contrast, we focused our research on the development of radiological evidence for intestinal damage because diagnosis by imaging is crucial for the assessment of patients with acute mesenteric ischemia. Thus far, the chronological sequence of early changes that follow mesenteric ischemia and reperfusion has not been described, and the

**Table 2** Magnetic resonance imaging results and differences between the two groups

Time	1-h	2-h	4-h	8-h
Group I percentages				
Free fluid	100%	100%	100%	100%
Free gas	0%	0%	0%	0%
HRI	0%	100%	100%	100%
PI	0%	0%	100%	100%
Bowel wall thinning	0%	100%	100%	100%
Bowel wall thickening	0%	0%	0%	0%
Wall signal (T2-W sequences)	0%	0%	100%	100%
Wall pneumatosis	0%	0%	0%	0%
Group II percentages				
Free fluid	100%	100%	100%	100%
Free gas	0%	0%	0%	0%
HRI	0%	100%	100%	100%
PI	0%	0%	100%	100%
Bowel wall thinning	0%	0%	0%	0%
Bowel wall thickening	0%	100%	100%	100%
Wall signal (T2-W sequences)	100%	100%	100%	100%
Wall pneumatosis	0%	0%	0%	0%
Differences between the two groups				
Free fluid	No	No	No	No
Free gas	No	No	No	No
HRI	No	No	No	No
PI	No	No	No	No
Bowel wall thinning	No	Yes	Yes	Yes
Bowel wall thickening	No	Yes	Yes	Yes
Wall signal (T2-W sequences)	Yes	Yes	No	No
Wall pneumatosis	No	No	No	No

HRI: Hypotonic reflex ileus; PI: Paralytic ileus.

role of MRI diagnosis is still widely debated.

Intestinal ischemia occurs when there is an interruption of the blood supply to the bowel, or when there is inadequate organ perfusion due to conditions of shock or hypovolemia. The initial damage caused by ischemia could be worsened by oxidative damage during reperfusion. This clinical entity is known as ischemia/reperfusion (I/R) injury<sup>[15]</sup>. Previous studies on mesenteric I/R in felines reported that tissue lesions produced during reperfusion were greater than those produced during ischemia<sup>[16]</sup>. Despite decades of research in this area, I/R injury remains a clinically challenging problem<sup>[17-22]</sup>. Patients are particularly susceptible to the damaging effects of increased neutrophil activation following intestinal I/R<sup>[23]</sup>. Consequently, many cases of intestinal I/R develop into shock, multiple organ failure, and death<sup>[24,25]</sup>.

The absence of reperfusion after acute SMA occlusion is characterized by clear macroscopic differences compared to AAMI with reperfusion. In the first case, there is a pale mesentery with thinning of mesenteric vessels and spasm of the jejunal loops due to the lack of blood flow and the contraction of the semilunar folds. With reperfusion, there is vascular congestion of the mesentery and bowel wall related to hypoxic damage of endothelial cells and subsequent blood extravasation following restoration of blood flow. These findings are in agreement with results obtained by previous authors<sup>[24-26]</sup>.

Analyzing MRI signal characteristics, we found that

the presence of free fluid in the abdomen (from the first hour) and HRI (from the second hour) are common and early findings in AAMI. It has been reported that a small amount of fluid is already visible 30 min after the ischemic event<sup>[24]</sup>; however, another study, in a porcine model, reported that free fluid is a late finding (detectable after 12 h)<sup>[25]</sup>. The presence of ascites is reported in up to 80% of cases<sup>[26]</sup>. HRI is a condition that occurs before PI, and it is considered an early reaction to the ischemic insult.

From the fourth hour, MRI also identified the presence of dilated loops with gas-fluid stasis (PI), reported in the literature in up to 65% of cases<sup>[25,27-29]</sup>, and a hyper-intense signal from the bowel wall in some loops in T2-W sequences. These four findings (free fluid, HRI, dilated loops with gas fluids and bowel wall hyper-intensity) were present in all rats with mesenteric ischemia, with or without reperfusion.

We found important differences between the 2 mechanisms (mesenteric ischemia with or without reperfusion). First of all, the signal intensity of the wall in T2-W sequences: in those rats without reperfusion the signal intensity is normal at the first and second hour and becomes hyper-intense at the fourth and eighth. On the contrary, when we cause a reperfusion the hyper-intense signal is clearly visible from the first hour due to interstitial edema. Another MRI finding is the presence of bowel wall thickening due to blood extravasation and edema, which is visible from the second hour in rats with reperfusion but never found in rats without reperfusion. Bowel wall thickening was previously found in an animal model of mesenteric venous ischemia<sup>[30]</sup>, suggesting that reperfusion produces hemorrhagic damage.

The early bowel wall thickening and hyper-intensity of the wall in T2-W sequence images may be considered signs of acute mesenteric ischemia with reperfusion. The presence of hyper-intense signal in T2-W images may be attributed to the edema of the submucosa caused by the release of vasoconstrictor amines that attract fluid from the bowel lumen<sup>[24,31]</sup>. The absence of mucosal thickening in arterial occlusion without reperfusion is interesting: indeed, the presence of wall thickening has been considered the most important feature of acute mesenteric ischemia<sup>[32]</sup> (with a prevalence of up to 96%). Nonetheless, we found that the wall thickening was only visible when the occlusion was followed by reperfusion. Further, our results showed that bowel wall thinning, detectable from the second hour after the SMA occlusion, is an extremely specific sign of occlusion without reperfusion. This finding was never detected in the cases with reperfusion. In the literature, bowel wall thinning seems to be heavily underestimated, as it is described in only 5% of cases<sup>[33]</sup>.

The treatment of AAMI without reperfusion is significantly different from treatment of AAMI with reperfusion<sup>[34]</sup>. Because mesenteric ischemia with and without reperfusion have different therapeutic approaches, the power to distinguish between these conditions may have a great clinical importance<sup>[35,36]</sup>. Additionally, the radiological findings of mesenteric ischemia have different cours-

es depending on whether the ischemic event followed by reperfusion: bowel wall thickening is a specific sign of reperfusion damage; acute SMA occlusion without reperfusion is characterized by bowel wall thinning. Although computer tomography remains a valid diagnostic tool for the visualization of mesenteric ischemia and for the evaluation of its damage, our study results suggest that MRI, which does not require the use of a contrast medium or ionizing radiation, can play an important role in the early diagnosis of this condition.

Because our model has some limitations, further investigation is necessary to verify whether these results can be translated to human beings. The first limitation is that we used a complete acute occlusion of the SMA, but in human beings it is possible to find partial occlusions. Second, the vessel ligation was performed at the vessel origin; this represents a potential bias because distal occlusions can occur. Other limitations include the small number of animals included in this preliminary report and the lack of histological analysis.

## COMMENTS

### Background

Acute arterial mesenteric ischemia is a life threatening vascular condition caused by the lack of arterial flow to the bowel, which can be occlusive or non-occlusive in origin. The initial damage caused by ischemia could be further worsened by oxidative damage during reperfusion. This entity is known as ischemia/reperfusion (I/R) injury. An early diagnosis of this condition is crucial to ensure a good outcome for the patient.

### Research frontiers

Although computer tomography (CT) remains a valid diagnostic tool for the visualization of mesenteric ischemia and for the evaluation of its damage, magnetic resonance imaging (MRI), which does not require the use of a contrast medium or ionizing radiation, can play an important role in the early diagnosis of this condition.

### Innovations and breakthroughs

Despite decades of research in this area, ischemia/reperfusion injury remains a clinically challenging problem. Patients are particularly susceptible to the damaging effects of increased neutrophil activation following intestinal ischemia/reperfusion. To ensure a good prognosis to these patients an early diagnosis is requested and the prerequisite to do it consists in the knowledge of the timing of the lesions. Important differences between the 2 mechanisms (mesenteric ischemia with or without reperfusion) were found at MRI, regarding the timing of the following findings: the signal intensity of the wall in T2-W sequences and the presence of bowel wall thickening due to blood extravasation and edema. Although CT remains the gold standard in the evaluation of acute mesenteric ischemia, our results suggest that MRI, which does not require the use of a contrast medium or ionizing radiation, could in future play an important role in the early diagnosis of this condition.

### Applications

This study suggests the following points: (1) The reperfusion of the mesenteric region, after 1 h of induced acute arterial ischemia, is characterized by early bowel wall hyperintensity in T2-W sequences, from the first hour, and bowel wall thickening, from the second hour; (2) Bowel wall thinning is a specific sign of acute superior mesenteric artery occlusion without reperfusion, detectable from the second hour; and (3) MRI can assess the various pathological changes that occur in the small bowel after induced acute arterial mesenteric ischemia with and without reperfusion.

### Terminology

Acute arterial mesenteric ischemia/infarction: lack of blood flow to the small bowel caused by a pathologic constriction or obstruction of its blood vessels, or an absence of blood circulation that can lead to ischemic damage of the bowel (involving part of the wall) or infarction (full thickness necrosis). 7T microMRI:

Non-invasive preclinical studies method of demonstrating internal anatomy based on the principle that atomic nuclei in a strong magnetic field (7 Tesla) absorb pulses of radiofrequency energy and emit them as radiowaves which can be reconstructed into computerized images.

### Peer review

The paper presents some new methodology concerning the detection of subtle changes that occurs during the acute arterial mesenteric ischemia and ischemia-reperfusion pathological process. The paper is well designed and the results are meaningful and conceivable.

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## Risk factors for hepatocellular carcinoma in patients with drug-resistant chronic hepatitis B

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### Abstract

**AIM:** To investigate the risk factors and characteristics of hepatocellular carcinoma (HCC) in the patients with drug-resistant chronic hepatitis B (CHB).

**METHODS:** A total of 432 patients with drug-resistant CHB were analyzed retrospectively from January 2004 to December 2012. The patients were divided into two groups: the HCC group ( $n = 57$ ) and the non-HCC group ( $n = 375$ ). Two groups compared using logistic regression for various patients and viral characteristics in order to identify associated risk factors for HCC. Secondly, patient and tumor characteristics of HCC patients with naïve CHB (N group,  $n = 117$ ) were compared to the HCC group (R group,  $n = 57$ ) to identify any difference in HCC characteristics between them.

**RESULTS:** A significant difference was found for age,

platelet count, alpha-fetoprotein (AFP), positivity of HBeAg, seroconversion rate of HBeAg, virologic response, the Child-Pugh score, presence of rtM204I, and the duration of antiviral treatment in non-HCC and HCC group. Cirrhosis, age ( $> 50$  years), HBeAg (+), virologic non-responder status, and rtM204I mutants were independent risk factors for the development of HCC. The R group had lower serum C-reactive protein (CRP) and AFP levels, earlier stage tumors, and a shorter mean tumor surveillance period than the N group. However, the total follow-up duration was not significantly different between the two groups.

**CONCLUSION:** 13.2% of patients with drug-resistant CHB developed HCC. Age, cirrhosis, YIDD status, HBeAg status, and virologic response are associated with risk of HCC. Patients with drug-resistant CHB and these clinical factors may benefit from closer HCC surveillance.

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**Key words:** Carcinoma; Hepatocellular; Hepatitis B; Drug resistance; Risk factors; Characteristics

**Core tip:** There are few studies on hepatocarcinogenesis in patients with drug-resistant chronic hepatitis B (CHB) or on the characteristics of tumors arising from drug-resistant CHB. In the present study, the cumulative incidence rate of hepatocellular carcinoma (HCC) in patients with drug-resistant CHB was 4.6%, 6.9%, 8.87%, and 11.8% at the end of 1, 2, 3, and 5 years, respectively. Additionally, cirrhosis, age  $> 50$  years, HBeAg (+), YIDD mutations, and a virologic non-responder status were independent risk factors for the development of HCC in CHB patients with drug resistance. Furthermore, there was a trend of poorer survival in patients with HCC arising from resistant CHB than in patients with HCC arising from naïve CHB.

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## INTRODUCTION

Chronic hepatitis B (CHB) is an important cause of morbidity and mortality worldwide<sup>[1,2]</sup>. The main goals of therapy for CHB patients are to prevent disease progression and to avoid the development of liver failure and hepatocellular carcinoma (HCC)<sup>[3]</sup>.

The introduction of oral nucleos(t)ide analogue (NA) therapy during the last two decades has revolutionized the CHB treatment<sup>[4,5]</sup>.

Although nucleoside/nucleotide analogues (NAs) are very effective at inhibiting HBV reverse transcriptase, the long-term use of NAs leads to the development of drug resistance. The risk of developing lamivudine (LAM) resistance is 14%-32% in the first year and up to 70% by the fifth year. For LAM resistance, the signature rtM204V/I and rtL180M mutations occur in more than 70% of CHB patients<sup>[6,7]</sup>. The rtM204V/I, a mutation located at the catalytic YMDD motif<sup>[8-10]</sup>, and the rtL180M mutations serve as compensatory mutations<sup>[10]</sup>. The rtA181T mutation has also been reported in a substantial proportion of LAM-resistant patients<sup>[9]</sup>. The risk of developing adefovir (ADV) resistance is 2%-3% and 28%-29% by the second and fifth year of monotherapy treatment in naïve patients, respectively<sup>[11,12]</sup>. The major ADV-resistant mutants were rtN236T and rtA181T/V<sup>[13]</sup>. Resistance to entecavir (ETV) is rare when being used to treat naïve patients (1.5% by the fifth year)<sup>[5]</sup>. However, in the presence of the rtM204I/V mutation, ETV resistance can occur if the rtI169T, rtT184A/F/G/I/L/S, rtS202G/I, or rtM250V mutation also exist<sup>[14,15]</sup>.

It has been demonstrated that in patients with compensated cirrhosis, LAM therapy significantly reduces the risk of liver failure and HCC<sup>[16]</sup>. However, in patients who have developed LAM resistance, this beneficial effect is drastically compromised<sup>[17]</sup>. One study reported that old age, male gender, family history of HCC, HBeAg positivity, genotype C, and increased levels of ALT, HBV DNA and HBsAg were risk factors for the development of HCC in chronic hepatitis B patients<sup>[18]</sup>. However, there are few studies on hepatocarcinogenesis in patients with drug-resistant CHB.

Therefore, we determined the risk factors for the development of HCC in patients with drug-resistant CHB. We also compared the tumor characteristics between HCC patients with drug-resistant CHB and HCC patients with CHB who were treated with antivirals.

## MATERIALS AND METHODS

### Patients and methods

Six hundred and forty-one patients with a documented

CHB mutation who had experienced a viral breakthrough at our institution (Chonnam National University Hospital, Gwangju, Korea) between January 2004 and December 2012 were selected for this retrospective study. The 209 patients who were excluded those whose entire set of laboratory data were not available at the end of the follow-up period, those who were co-infected with HIV, hepatitis C or hepatitis D, those who were chronic alcohol drinkers, those who had poor medication compliance and those who had been diagnosed with HCC before the CHB mutation occurred. In total, the data of 432 CHB patients (most of them, genotype C, we did not check the genotype of all patients ( $n = 90/432$ ), but 100% of the 90 patients whose genotypes were checked were genotype C) with drug resistance were finally evaluated (Figure 1). To determine the risk factors for the development of HCC in patients with drug-resistant CHB, the patients were divided into two groups according to the occurrence of HCC (57 patients with HCC *vs* 375 patients without HCC).

To compare the tumor characteristics between the HCC patients with drug-resistant CHB (R group) and the HCC patients with CHB treated with antivirals, we selected 119 HCC patients who had received first line antiviral treatment without evidence of drug resistance (N group) during the same period (Figure 1).

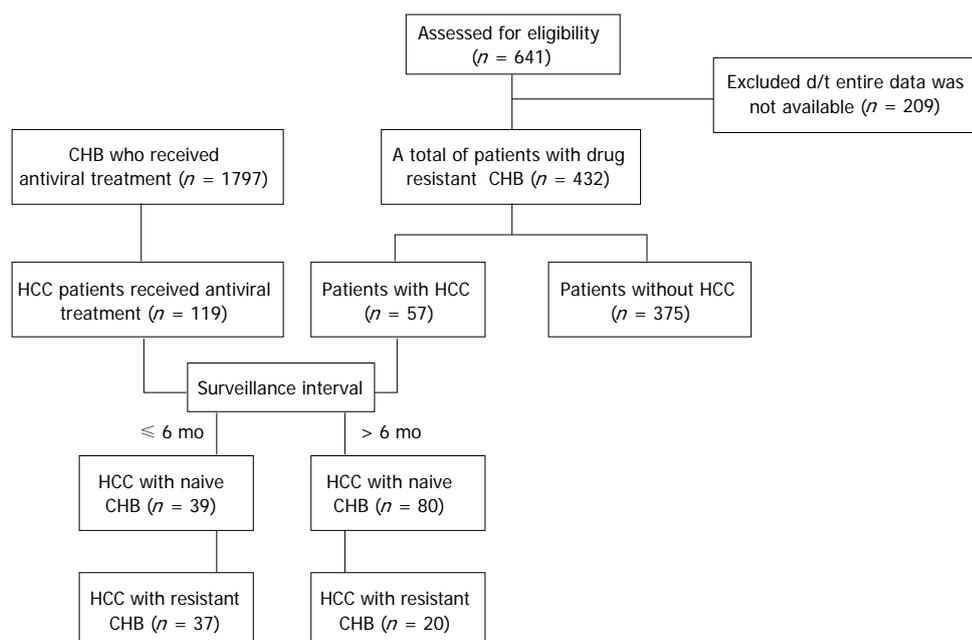
The diagnosis of HCC was based on the following guidelines proposed by the Korea Liver Cancer Study Group and the National Cancer Center<sup>[19]</sup>: (1) nodules > 2 cm in diameter with a typical pattern of HCC in one imaging study or AFP levels > 200 ng/mL; and (2) nodules between 1 and 2 cm in diameter with a coincidental typical vascular pattern in two imaging studies. If these criteria were not met, biopsies were performed. Clinical staging was based on the modified UICC tumor-node-metastasis classification and the Cancer of the Liver Italian Program (CLIP) scores<sup>[19,20]</sup>.

Clinicoradiological and virological variables and the occurrence of HCC were compared between the two groups. The clinicoradiological variables were age, sex, the Child-Pugh score, blood chemistry, AFP level, and the duration of the antiviral treatment. The virological variables were positivity of HBeAg, seroconversion of HBeAg, HBV DNA level, virologic response rate, and mutant type. Informed consent was obtained from all patients about the nature and purpose of the treatment modalities.

### Marker of HBV infection

The hepatitis B e antigen (HBeAg) and antibodies to HBeAg (anti-HBe) were determined by commercially available radioimmunoassay systems (Abbott Laboratories, Abbott Park, IL, United States). The serum HBV DNA was quantified by the TaqMan<sup>®</sup> real time polymerase chain reaction (PCR) system (Applied Biosystems, Foster city, CA, United States). The lower limit of detection was 100 copies/mL.

Mutations were identified at the baseline using restriction fragment mass polymorphism (RFMP) analysis.



**Figure 1 Study patient flow.** HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis B.

### Diagnosis and surveillance of HCC

All patients were examined for HCC by abdominal ultrasonography (US) and/or abdominal computed tomography (CT) ± serum alpha-fetoprotein (AFP) examination every 3-12 mo. If HCC was suspected, additional procedures, such as CT, magnetic resonance imaging (MRI), angiography, and US guided biopsy, were performed as necessary to confirm the diagnosis.

### Definitions

A primary non-response was defined as a less than 1 log copy/mL decrease in the HBV DNA level from the baseline at 3 mo of treatment. A partial virologic response was defined as a ≥ 1 log copy/mL decline in the HBV DNA level from the baseline, but with a detectable load, at week 24. A complete virologic response was defined as an undetectable HBV DNA level by a sensitive PCR assay<sup>[21]</sup>.

The R group was defined as patients with HCC arising from drug-resistant CHB, and the N group was defined as patients with HCC arising from CHB receiving first line antiviral treatment without evidence of drug resistance.

Subgroup analysis according to tumor surveillance interval, the R' group was defined as patients with HCC arising from drug-resistant CHB who underwent frequent tumor surveillance (≤ 6 mo), and the N' group was defined as patients with HCC arising from CHB treated with antivirals who underwent frequent tumor surveillance (≤ 6 mo).

### Statistical analysis

The parametrical data were expressed as the means ± SD when a normal distribution was assumed and were expressed as a median (range) when a normal distribution was not assumed to be present. The group comparisons

were performed using the Pearson's  $\chi^2$  test and Mann-Whitney *U*-test.

Logistic regression analysis was performed to evaluate the factors for the development of HCC. Factors that were significant in the univariate analysis were entered into a stepwise multivariate analysis to find the most significant risk factors. The hazard function data were estimated using the Kaplan-Meier curve and compared using the log rank test. A *P*-value of less than 0.05 was considered to be statistically significant. We performed statistical analysis using SPSS 20.0 (SPSS Inc., Chicago, United States).

## RESULTS

### Baseline characteristics of patients with drug-resistant CHB

The median follow-up duration was 49.3 mo (range: 0-137 mo). The mean age of the patients was 48.48 (range 16-81) years. The mean duration of the antiviral treatment was 30.55 mo (4-120 mo). There were 309 men (71.5%) and 123 women (28.5%) included in the study. Additionally, 127 patients (29.4%) had cirrhosis; 350 patients (81%) were positive for HBeAg; 118 patients (27.3%) were rtM204I-positive; 54 patients (12.5%) were rtM204I/rtL180 M-positive; 39 patients (9.0%) were rtM204I+V/rtL180 M-positive; 125 patients (28.9%) were rtM204 V/rtL180 M-positive; 25 patients (5.8%) were rtA181T-positive; 2 patients (0.4%) were rtA181T/V-positive; 12 patients (2.7%) were rtN236T-positive; and 57 patients (13.2%) were positive for other mutations.

The clinical characteristics of the two groups (HCC-positive group vs HCC-negative group) are shown in Table 1. There were no significant differences between the two groups for gender, initial HBV DNA level, serum aspartate aminotransferase (AST) level, alanine

**Table 1** Characteristics of patients with drug-resistant chronic hepatitis B stratified by hepatocellular carcinoma status *n* (%)

Variables	Patients with HCC ( <i>n</i> = 57)	Patients without HCC ( <i>n</i> = 375)	<i>P</i> -value
Gender (male)	42 (73.7)	267 (71.2)	0.755
Age (mean, yr)	57.44 ± 8.35	47.03 ± 12.25	< 0.001
Presence of cirrhosis	42 (73.7)	85 (22.7)	< 0.001
HBeAg positivity	36 (63.2)	314 (83.7)	0.003
Seroconversion of HBeAg	3 (5.3)	78 (20.8)	0.022
Initial HBV DNA (< 2000 IU)	2 (3.5)	27 (7.2)	0.61
Initial HBV DNA (> 20000 IU)	46 (80.7)	314 (83.7)	0.162
Complete virologic response	11 (19.3)	135 (36)	0.02
Partial virologic response	31 (54.4)	271 (72.3)	0.012
Platelet count (10 <sup>3</sup> /mm <sup>3</sup> ) (median, range)	103 (35-380)	164.5 (34-330)	< 0.001
AST U/L (median, range)	79.5 (22-707)	63 (14-1494)	0.126
ALT U/L (median, range)	86.5 (11-590)	82 (9-2280)	0.770
AFP IU/mL (median, range)	7.58 (1.54-1890)	2.8 (0.65-980)	< 0.001
Child-Pugh score	5.51 ± 0.85	5.12 ± 0.71	0.002
Viral mutation			
rtM204I	25 (43.9)	93 (24.8)	0.004
rtM204I/rtL180M	4 (7)	50 (13.3)	0.098
rtM204V/rtL180M	14 (24.6)	111 (29.6)	0.492
rtM204V + I/rtL180M	3 (5.3)	36 (9.6)	0.451
rtA181T	3 (5.3)	22 (5.9)	1
rtA181T/V	1 (1.8)	1 (0.26)	0.224
Duration of antiviral treatment (mo)	24.31 ± 15.28	31.43 ± 19.43	0.004

HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; HBV: Hepatitis B virus.

aminotransferase (ALT) level, or total bilirubin or albumin. A significant difference was found for age, platelet count, AFP, positivity of HBeAg, seroconversion rate of HBeAg, virologic response, the Child-Pugh score, presence of rtM204I, and the duration of antiviral treatment.

### Clinical and virologic factors associated with HCC occurrence

The univariate analysis showed that cirrhosis, age > 50 years, partial virologic response, complete virologic response, the rtM204I (YIDD) mutation, HBeAg (+), platelet count, the Child-Pugh score, and the duration of antiviral treatment were associated with the development of HCC. The multivariate analysis showed that cirrhosis, age > 50 years, negative complete virologic response, HBeAg (+), and the rtM204I (YIDD) mutation were independent risk factors for the development of HCC (Table 2).

### Cumulative incidence of HCC in patients with drug-resistant CHB

The cumulative hepatocarcinogenesis rate in patients with

**Table 2** Multivariate analysis of the clinical and virologic factors associated with hepatocellular carcinoma occurrence

Variables	HR	95%CI	<i>P</i> -value
Presence of cirrhosis	8.196	3.623-18.518	< 0.01
Age > 50 yr	3.426	1.445-8.123	< 0.01
Complete virologic response	0.164	0.054-0.276	< 0.01
Positivity of HBeAg	2.893	1.143-7.327	< 0.05
Presence of rtM204I mutation	3.412	1.54-6.440	< 0.01

drug-resistant CHB was 4.6%, 6.9%, 8.87%, and 11.8% at the end of 1, 2, 3, and 5 years, respectively.

Kaplan-Meier analysis for the incidence rate of HCC in patients with drug-resistant CHB stratified by the patient and viral factors identified in multivariate analysis is shown in Figure 2.

The cumulative occurrence rate of HCC in cirrhotic patients with drug-resistant CHB was 3.93%, 5.55%, 6.71%, and 9.02% at the end of 1, 2, 3 and 5 years, respectively. The cumulative occurrence rate of HCC in HBeAg (+) patients was 3.0%, 5.09%, 6.01%, and 7.87% at the end of 1, 2, 3 and 5 years, respectively. The cumulative occurrence rate of HCC in rtM204I (YIDD) mutant patients was 2.3%, 3.0%, 4.16%, and 6.01% at the end of 1, 2, 3 and 5 years, respectively. The cumulative occurrence rate of HCC in patients greater than 50 years of age was 6.9%, 11.5%, 20.8%, and 23.1% at the end of 1, 2, 3 and 5 years, respectively.

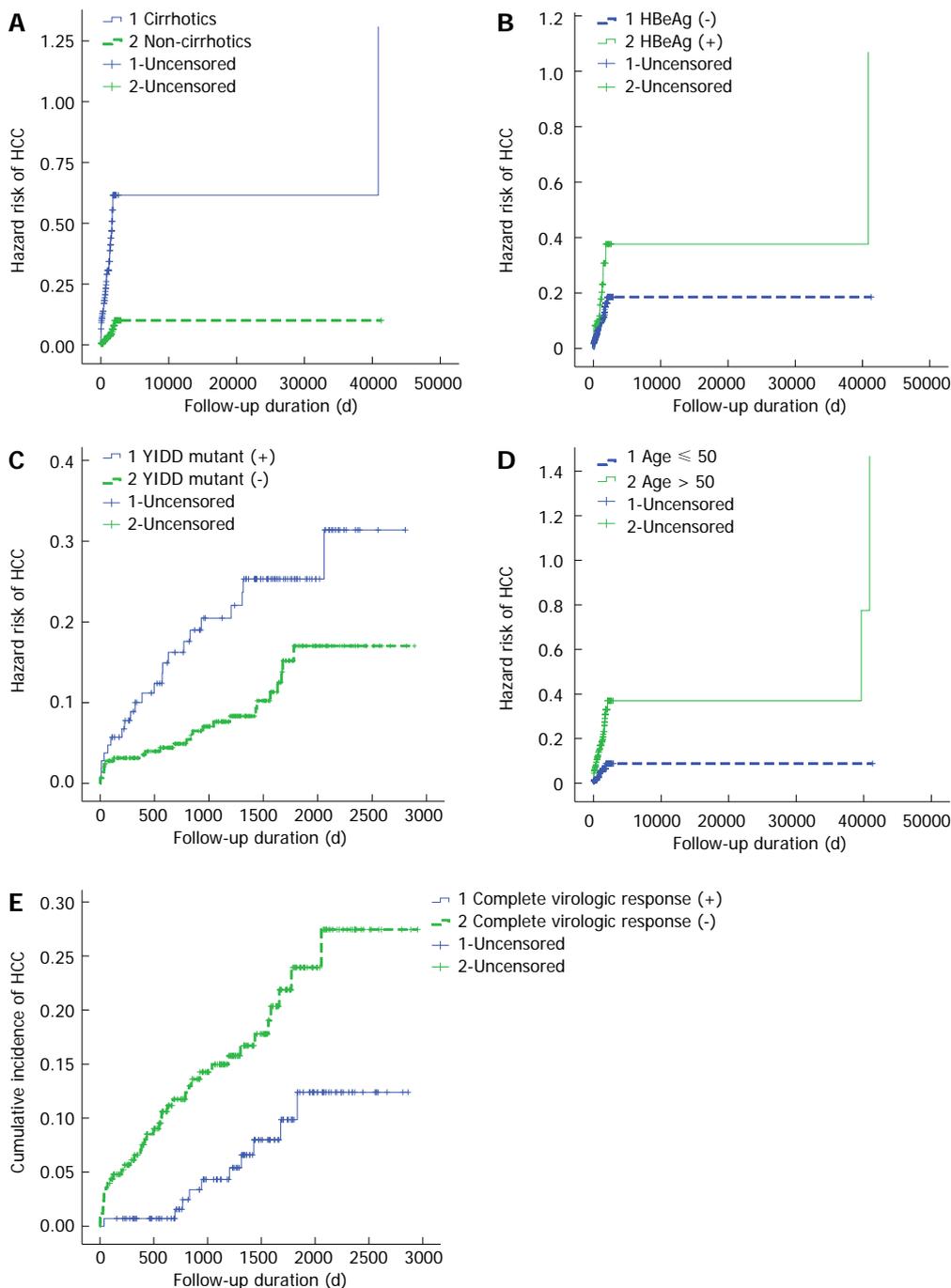
The cumulative occurrence rate of HCC in virologic non-responder patients was 2.3%, 4.6%, 11.5%, and 23.1% at the end of 1, 2, 3 and 5 years, respectively.

### Tumor characteristics of patients with HCC and drug-resistant CHB compared to patients with HCC and naive CHB

To compare the tumor characteristics between HCC patients with drug-resistant CHB (R group) and HCC patients with CHB treated with antivirals, we selected 119 HCC patients with CHB who received antiviral treatment (N group). The clinical characteristics of the two groups (R group *vs* N group) are shown in Table 3. There were no significant differences between the two groups for sex, age, presence of cirrhosis, tumor type, serum ALT, the Child-Pugh score, or the total follow-up duration. A significant difference was found for portal vein thrombosis, tumor stage (CLIP score, modified UICC), distant metastasis, platelet count, serum AST, C-reactive protein (CRP), AFP, and the mean tumor surveillance interval. The R group had lower serum CRP and AFP levels and earlier stage tumors than the N group. The total follow-up duration was not significantly different between the two groups.

### Subgroup analysis according to tumor surveillance interval

The R group had tumors of an earlier stage and a lower AFP than the N group because of frequent tumor surveillance. Therefore, we performed a subgroup analysis to



**Figure 2 Cumulative risk.** A, B: Hepatocellular carcinoma (HCC) in cirrhotic patients and in HBeAg (+) patients with drug-resistant chronic hepatitis B (CHB) (log rank test,  $P < 0.001$ ,  $P = 0.008$ , respectively); C, D: HCC in YIDD patients and in patients  $> 50$  years of age with drug-resistant CHB (log rank test,  $P = 0.007$ ,  $P = 0.001$ , respectively); E: HCC in complete virologic responders with drug-resistant CHB (log rank test,  $P = 0.003$ ).

exclude the frequent tumor surveillance effect on tumor prognosis. Patients were divided into two groups ( $> 6$  mo *vs*  $\leq 6$  mo) according to the interval of their surveillance.

Thirty-seven patients with drug-resistant CHB (R' group) and 39 patients with naïve CHB who received antiviral treatment (N' group) (surveillance interval  $\leq 6$  mo) were selected. The clinical characteristics of the two groups (R' group *vs* N' group) are shown in Table 4. There were no significant differences between the two groups for most variables analyzed. A significant difference was found in the serum CRP and AFP levels, the

duration of the antiviral treatment, and the total follow-up duration. The R' group had lower serum CRP and AFP levels than the N' group at a similar stage. However, the total follow-up duration was shorter in the R' group than the N' group.

In addition, 20 patients with drug-resistant CHB and 80 patients with CHB who received antiviral treatment (surveillance interval  $> 6$  mo) were selected. There were no significant differences between the two groups for most of the variables analyzed, including tumor stage, serum CRP and AFP levels, and total follow-up duration.

**Table 3 Comparison of tumor characteristics between patients with hepatocellular carcinoma with resistant chronic hepatitis B (R group) and hepatocellular carcinoma with chronic hepatitis B treated with antivirals (N group) *n* (%)**

Variables	R group ( <i>n</i> = 57)	N group ( <i>n</i> = 119)	<i>P</i> -value
Gender (male)	42 (73.7)	100 (84)	0.158
Age (mean, yr)	57.44 ± 8.35	55.37 ± 10.61	0.161
Presence of cirrhosis	55 (96.5)	117 (98.3)	1
Portal vein thrombosis	8 (14)	43 (36.1)	0.004
Vascular invasion	20 (35.1)	47 (39.5)	0.623
Multi-nodular tumor type	21 (36.8)	55 (46.2)	0.259
CLIP score	0.75 ± 0.85	1.67 ± 1.43	< 0.001
Modified UICC stage (< IVA)	50 (87.7)	73 (61.3)	< 0.001
Modified UICC stage (I / II / III / IVA / IVB)	13/18/19/6/1	18/34/21/28/18	
LN involvement	1 (2.1)	14 (11.7)	0.071
Distant metastasis	0 (0)	13 (10.9)	0.019
AST U/L (median, range)	48 (20-415)	61 (16-481)	0.022
ALT U/L (median, range)	39 (8-532)	44 (3-203)	0.616
Platelet count (10 <sup>3</sup> /mm <sup>3</sup> ), (median, range)	103 (23-380)	126 (24-426)	0.009
CRP (mg/dL) (median, range)	0.34 ± 0.37	1.71 ± 2.75	< 0.001
AFP IU/mL (median, range)	48 (2-40591)	107 (2-50000)	0.003
Child-Pugh score	5.51 ± 0.85	5.91 ± 1.32	0.344
Duration of anti-viral Tx. (mo)	20 (0-72)	6 (1-276)	0.001
Mean tumor surveillance period (mo)	6.35 ± 1.69	8.71 ± 2.86	< 0.001
Total follow-up duration (d)	842.51 ± 702.57	801.74 ± 713.24	0.721

UICC: Union Internationale Contre le Cancer; CLIP: Cancer of the Liver Italian Program; LN: Lymph node; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; CRP: C-reactive protein.

## DISCUSSION

Antiviral therapy was shown to significantly reduce the risk of liver failure and HCC in patients with compensated cirrhosis and CHB<sup>[16]</sup>. However, the development of LAM-resistance significantly compromises the effectiveness of this strategy<sup>[17]</sup>. Until now, there have been few studies about hepatocarcinogenesis in patients with drug-resistant CHB.

Data regarding the characteristics of HCC that arise in patients with drug-resistant CHB are also lacking.

Many countries rely on LAM as first-line antiviral therapy for CHB, and over time many patients will develop drug resistance.

Therefore, it is imperative that clinical and virologic factors associated with the development of HCC in this group of patients be delineated.

In our study, we demonstrated that the cumulative incidence rate of HCC in patients with drug-resistant CHB was 4.6%, 6.9%, 8.87%, and 11.8% at the end of 1, 2, 3, and 5 years, respectively, and that cirrhosis, age >

**Table 4 Comparison of tumor characteristics between patients with hepatocellular carcinoma with resistant chronic hepatitis B (R' group) and hepatocellular carcinoma with chronic hepatitis B treated with antiviral treatment (N' group): subgroup analysis based on tumor surveillance interval (≤ 6 mo) *n* (%)**

Variables	R' group ( <i>n</i> = 37)	N' group ( <i>n</i> = 39)	<i>P</i> -value
Gender (male)	27 (73)	31 (79.5)	0.594
Age (mean, yr)	55.24 ± 7.94	56.08 ± 10.3	0.695
Presence of cirrhosis	36 (97.3)	39 (100)	1
Portal vein thrombosis	5 (13.5)	8 (20.5)	0.546
Vascular invasion	11 (29.7)	8 (20.5)	0.431
Multi-nodular tumor type	27 (73)	27 (69.2)	0.803
CLIP score	0.59 ± 0.72	0.97 ± 0.98	0.061
Modified UICC stage (< IVA)	34 (91.9)	35 (89.7)	1.0
Modified UICC stage (I / II / III / IVA / IVB)	11/11/12/3/0	11/15/9/2/2	
LN involvement	0 (0)	2 (5.1)	0.496
Distant metastasis	0 (0)	1 (2.6)	1
AST U/L (median, range)	48 (20-141)	52 (16-481)	0.283
ALT U/L (median, range)	38 (8-199)	44 (11-150)	0.14
Platelet count (10 <sup>3</sup> /mm <sup>3</sup> ) (median, range)	101 (23-232)	131 (24-260)	0.059
CRP (mg/dL) (median, range)	0.3 (0-2)	0.5 (0-16)	0.029
AFP IU/mL (median, range)	27 (2-737)	52 (3-16644)	0.039
Child-Pugh score	5.51 ± 0.85	5.91 ± 1.32	0.982
Duration of anti-viral Tx	21.5 (8-72)	7 (1-60)	< 0.001
Total follow-up duration	791.95 ± 643.06	1114.23 ± 646.84	0.033

UICC: Union Internationale Contre le Cancer; CLIP: Cancer of the Liver Italian Program; LN: Lymph node; ALT: Alanine aminotransferase; AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; CRP: C-reactive protein.

50 years, HBeAg (+), the rtM204I (YIDD) mutation, and virologic non-responder status were independent risk factors for the development of HCC in CHB patients with drug resistance.

Previous studies primarily identified the rate of hepatocarcinogenesis with varying results. Akuta *et al*<sup>[22]</sup> reported a cumulative hepatocarcinogenesis rate in LAM resistant hepatitis B genotype C patients of 2.2%, 5.9%, and 8.1% at the end of 1, 2, and 3 years, respectively, and they indicated that these hepatocarcinogenesis rates were similar to those in cirrhosis patients who had not received antiviral therapy (namely, the high-risk group for HCC development)<sup>[23]</sup>. Hosaka *et al*<sup>[24]</sup> reported that HCC developed in 18 of the 247 (7.3%) patients who had received adefovir add-on lamivudine during a 5-year period. Consistent with the results of other studies, the cumulative incidence rate of HCC in patients with drug-resistant CHB in our study was 4.6%, 6.9%, 8.87%, and 11.8% at the end of 1, 2, 3, and 5 years, respectively; however, our study had a larger number of cases than the other two studies.

Regarding the risk factors that influence the development of HCC, Hosaka *et al*<sup>[24]</sup> reported that AST > 70 IU/L, the rtM204I (YIDD) mutation, age > 50 years and cirrhosis were independent risk factors for the development of HCC. Additionally, Yeh *et al*<sup>[9]</sup> reported

that the rtA181T mutation, age > 50 years, and liver cirrhosis were significantly associated with the occurrence of HCC. In another study<sup>[22]</sup>, the cumulative HBV DNA non-detectable rate and ALT normalization rate were not significantly different with regards to the development of HCC. Our study showed, distinctly from other studies, that cirrhosis, age > 50 years, a negative complete virologic response, the rtM204I (YIDD) mutation, and HBeAg (+) were independent risk factors for the development of HCC. In our study, the rtA181T mutation and HBV DNA level at the time of the documentation of the drug mutation were not associated with the occurrence of HCC. Consistent with other studies on naïve CHB patients<sup>[18]</sup>, HBeAg (+) and a negative complete virologic response were associated with the development of HCC.

HCC arising from drug-resistant CHB (R group) had lower serum CRP and AFP levels and earlier stage tumors than HCC arising from naïve CHB (N group). Although the R group had an earlier stage tumors and lower serum CRP and AFP levels, the total follow-up duration was not significantly different between the two groups. We think these differences may be attributed to more frequent tumor surveillance in the R group. The R group may have had the same survival rates even if the R group had better prognostic factors, such as low tumor stage and low AFP and CRP levels compared to the N group. Therefore, we performed a subgroup analysis to exclude the frequent tumor surveillance effect on the tumor prognosis. The subgroup analysis showed that the patients with HCC arising from drug-resistant CHB who underwent frequent tumor surveillance ( $\leq 6$  mo) (R' group) had lower serum CRP and AFP levels than patients with HCC arising from CHB treated with antiviral treatment who underwent frequent tumor surveillance ( $\leq 6$  mo) (N' group) at the same stage. However, the total follow-up duration was shorter in the R' group than the N' group, paradoxically. Recent studies reported that an increased serum CRP level is associated with poor prognosis (tumor recurrence after a surgical resection, large tumor size, and poorly defined tumor type) of patients with HCC<sup>[25,26]</sup>. Additionally, serum AFP is well known as a prognostic factor for patients with HCC<sup>[27,28]</sup>. These findings suggest that HCC patients with naïve CHB may have a better prognosis than HCC patients with resistant CHB, irrespective of the tumor stage or serum CRP or AFP levels in the frequent tumor surveillance group. That is, patients with HCC arising from resistant CHB may have poorer survival than patients with HCC arising from naïve CHB. These findings may be explained by the R group having poorer liver function because of incomplete viral suppression during follow-up. In fact, hepatic failure was the main cause of death in the R group (data not shown). To us, this is the most striking finding.

However, in the HCC surveillance group, in which the surveillance exceeded 6 mo, there were no significant differences between the two subgroups for most of the variables analyzed, including tumor stage, serum CRP and AFP levels, and total follow-up duration.

The first limitation of our study is that it was a retrospective single center study. Therefore, our results may not be generalizable to other patient populations.

The second limitation is a single data point for various viral markers (*e.g.*, viral DNA) and laboratory values is another limitation since these factors are not static over time. Third, the relationship between risk factors, such as nucleotide substitution and the development of HCC, could not be presented. Fourth, we did not check the genotype of all patients ( $n = 90/432$ ), but 100% of the 90 patients whose genotypes were checked were genotype C. In South Korea, it is well known that the genotype of CHB patients is almost always genotype C.

Larger, prospective studies will be needed to confirm these findings.

In summary, frequent HCC surveillance may identify early HCC in these high-risk patients, and patients with HCC arising from resistant CHB may have poor survival, irrespective of tumor stage or serum CRP or AFP levels.

## COMMENTS

### Background

In patients with compensated cirrhosis, antiviral therapy significantly reduces the risk of liver failure and hepatocellular carcinoma (HCC). However, in patients who have developed drug resistance, this beneficial effect is drastically compromised. However, there are few studies on hepatocarcinogenesis in patients with drug-resistant chronic hepatitis B (CHB) or on the characteristics of tumors arising from drug-resistant CHB.

### Related publication

Hosaka *et al* reported that aspartate aminotransferase > 70 IU/L, the rtM204I (YIDD) mutation, age > 50 years and cirrhosis were independent risk factors for the development of HCC. Yeh *et al* reported that the rtA181T mutation, age > 50 years, and liver cirrhosis were significantly associated with the occurrence of HCC.

### Innovations and breakthroughs

Cirrhosis, age > 50 years, HBeAg (+), YIDD mutants, and virologic non-responder status were independent risk factors for the development of HCC in CHB patients with drug resistance, and there was a trend of poorer survival in patients with HCC arising from resistant CHB than in patients with HCC arising from naïve CHB.

### Applications

Frequent HCC surveillance may identify HCC early in these high-risk patients.

### Peer review

This is a large ( $n = 432$ ) monocenter study aimed at evaluating the relationship between drug-resistant HBV chronic infection and the development of HCC. It is well written.

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## Differential diagnosis of left-sided abdominal pain: Primary epiploic appendagitis vs colonic diverticulitis

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### Abstract

**AIM:** To investigate the clinical characteristics of left primary epiploic appendagitis and to compare them with those of left colonic diverticulitis.

**METHODS:** We retrospectively reviewed the clinical records and radiologic images of the patients who presented with left-sided acute abdominal pain and had computer tomography (CT) performed at the time of presentation showing radiological signs of left primary epiploic appendagitis (PEA) or left acute colonic diverticulitis (ACD) between January 2001 and December

2011. A total of 53 consecutive patients were enrolled and evaluated. We also compared the clinical characteristics, laboratory findings, treatments, and clinical results of left PEA with those of left ACD.

**RESULTS:** Twenty-eight patients and twenty-five patients were diagnosed with symptomatic left PEA and ACD, respectively. The patients with left PEA had focal abdominal tenderness on the left lower quadrant (82.1%). On CT examination, most (89.3%) of the patients with left PEA were found to have an oval fatty mass with a hyperattenuated ring sign. In cases of left ACD, the patients presented with a more diffuse abdominal tenderness throughout the left side (52.0% vs 14.3%;  $P = 0.003$ ). The patients with left ACD had fever and rebound tenderness more often than those with left PEA (40.0% vs 7.1%,  $P = 0.004$ ; 52.0% vs 14.3%,  $P = 0.003$ , respectively). Laboratory abnormalities such as leukocytosis were also more frequently observed in left ACD (52.0% vs 15.4%,  $P = 0.006$ ).

**CONCLUSION:** If patients have left-sided localized abdominal pain without associated symptoms or laboratory abnormalities, clinicians should suspect the diagnosis of PEA and consider a CT scan.

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**Key words:** Acute abdomen; Differential diagnosis; Appendix epiploica; Colonic diverticulitis; Multidetector computed tomography

**Core tip:** The clinical symptoms of primary epiploic appendagitis (PEA) and acute colonic diverticulitis (ACD) are similar in patients presenting with left-sided abdominal pain. In our study, the patients with PEA had well-localized abdominal tenderness, whereas those with ACD presented with slightly diffuse abdominal

tenderness. The patients with ACD showed fever, rebound tenderness, and leukocytosis more often than those with PEA. When patients have well-localized abdominal tenderness without associated systemic manifestation or laboratory abnormalities, clinicians should suspect a diagnosis of PEA and consider a computer tomography (CT) scan. The characteristic CT findings of PEA may enable clinicians to accurately diagnose the disease.

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## INTRODUCTION

Epiploic appendages are small pouches of peritoneum filled with fat and small vessels that protrude from the serosal surface of the colon<sup>[1-4]</sup>. Primary epiploic appendagitis (PEA) is an inflammation in the epiploic appendage caused by either torsion or spontaneous thrombosis of an appendageal draining vein<sup>[5-9]</sup>.

PEA is a rare cause of localized abdominal pain in otherwise healthy patients. The only clinical feature of PEA is focal abdominal pain and tenderness, without pathognomonic laboratory findings. Clinically, it can be often mistaken for either diverticulitis or appendicitis, and may be treated with antibiotic therapy or even surgical intervention.

Historically, the diagnosis of PEA had been made at diagnostic laparotomy, performed for presumed appendicitis or diverticulitis with complications<sup>[7,8,10,11]</sup>. With advancements in radiologic techniques, such as ultrasonography or computed tomography (CT), PEA can be distinguished preoperatively due to its characteristic radiologic findings, and it is already being diagnosed increasingly<sup>[8,12]</sup>.

Diverticulitis is the disorder most likely to be confused with PEA in a patient presenting with localized abdominal pain. In South Korea, as diverticulitis occurs much less in the left colon and PEA occurs quite frequently in the left colon; both diseases of the left colon remain difficult to differentiate.

Given that PEA is a benign and self-limited condition, the recognition of this diagnosis is important to clinicians to avoid unnecessary hospitalizations, antibiotic therapy, surgical interventions, and overuse of medical resources<sup>[7,10,13]</sup>. However, PEA cases are still infrequent and may often be missed even after imaging studies<sup>[8,10]</sup>.

There are no previous studies specifically designed to compare the clinical characteristics of left PEA with those of left acute colonic diverticulitis (ACD). The pres-

ent study was carried out to describe the clinical characteristics and characteristic CT findings of left PEA and to compare them with those of left ACD.

## MATERIALS AND METHODS

This study was performed on patients who presented with acute left-sided abdominal pain and diagnosed with left PEA or left ACD on CT findings at Konyang University Hospital from January 2001 to December 2011.

We retrospectively reviewed the clinical records and CT images of the study patients after obtaining approval from the institutional review board with regard to the clinical characteristics, presumed diagnosis before the imaging studies, laboratory findings, radiologic findings, and treatments. If data for specific findings were missing, they were not included in the final analysis.

All official CT scans were retrospectively reviewed by two radiologists to determine whether the imaging findings corresponded to PEA or ACD. We selected patients who were given the same diagnosis by two radiologists. The diagnosis of PEA was based on characteristic CT findings as shown below<sup>[10,11,14-16]</sup>: (1) ovoid fatty mass; (2) hyperattenuated ring sign; (3) disproportionate fat stranding; (4) bowel wall thickening with or without compression; (5) central hyperdense dot/line; and (6) lobulated appearance. The diagnosis of ACD was based on CT findings such as the presence of inflamed diverticula or thickened colonic wall more than 4 mm<sup>[7,16-18]</sup>.

The left colon was defined as the segment of colon under the splenic flexure, which included the descending and sigmoid colon. The size of PEA was the largest diameter on the radiologic findings. The shapes were oval, semicircular, and triangular.

We evaluated symptom recurrence in the patients with PEA by reviewing the records of subsequent visits. One patient with PEA was lost to follow-up.

### Statistical analysis

Statistical analysis was performed with SPSS, Windows version 18.0 (SPSS Inc., Chicago, IL, United States), using the  $\chi^2$  test and the Fisher's exact test. The averages were compared by using the *t*-test. A *P*-value of less than 0.05 was considered significant.

## RESULTS

### Clinical characteristics of patients with left primary epiploic appendagitis

There were 28 consecutive patients diagnosed as having left PEA on the CT reports and their clinical characteristics are shown in Table 1. The mean age (mean  $\pm$  SD) was 45.0  $\pm$  11.6 years (range, 24-65 years), and they were more common in males (ratio of males to females = 16: 12). All the patients had sudden onset of abdominal pain. Two patients (7.1%) showed nausea and vomiting, and fever up to 38.3 °C was present in two patients (7.1%). The abdominal tenderness was localized in the left lower

**Table 1 Clinical characteristics of patients with left primary epiploic appendagitis and left acute colonic diverticulitis**

	Lt. PEA (n = 28)	Lt. ACD (n = 25)	P-value
Mean age (yr)	45.0 ± 11.6	58.8 ± 16.3	0.001
Sex (male/female)	16/12	15/10	0.833
Body mass index (kg/m <sup>2</sup> )	24.8 ± 3.3	24.9 ± 3.0	0.921
Underlying disease (+)	6 (21.4)	10 (40.0)	0.142
Alcohol (+)	14 (50.0)	13 (52.0)	0.884
Smoking (+)	11 (39.3)	8 (32.0)	0.581
Sudden onset of abdominal pain (+)	28 (100.0)	25 (100.0)	NA
Duration of pain (d)	3.8 ± 5.3	4.6 ± 4.1	0.537
Nausea (+)	2 (7.1)	2 (8.0)	1.00
Vomiting (+)	2 (7.1)	2 (8.0)	1.00
Diarrhea (+)	0 (0.0)	3 (12.0)	0.098
Fever (+)	2 (7.1)	10 (40.0)	0.004
Location of abdominal tenderness			0.003
Focal	24 (85.7)	12 (48.0)	
Lt. lower quadrant	23 (82.1)	11 (44.0)	
Lt. upper quadrant	1 (3.6)	1 (4.0)	
Diffuse	4 (14.3)	13 (52.0)	
Rebound tenderness (+)	4 (14.3)	13 (52.0)	0.003
Palpable mass (+)	1 (3.6)	0 (0.0)	1.00

Values are presented as mean ± SD or n (%). PEA: Primary epiploic appendagitis; ACD: Acute colonic diverticulitis; NA: Not available.

**Table 2 Presumptive diagnoses prior to imaging studies n (%)**

Impression	Lt. PEA (n = 28)	Lt. ACD (n = 25)
PEA	7 (25.0)	0 (0.0)
ACD	16 (57.1)	15 (60.0)
Pelvic inflammatory disease	0 (0.0)	1 (4.0)
Ureter stone	1 (3.6)	0 (0.0)
Gastritis	2 (7.1)	0 (0.0)
Constipation	1 (3.6)	1 (4.0)
Appendicitis	1 (3.6)	1 (4.0)
Ischemic colitis	0 (0.0)	1 (4.0)
Cancer	0 (0.0)	1 (4.0)
Peritonitis	0 (0.0)	2 (8.0)
Enteritis (colitis)	0 (0.0)	3 (12.0)

PEA: Primary epiploic appendagitis; ACD: Acute colonic diverticulitis.

(82.1%) and left upper (3.6%) quadrant. Rebound tenderness was found only in four patients (14.3%), and one patient (3.6%) showed palpable mass. The presumptive clinical diagnoses after medical history and physical examinations were ACD (57.1%), PEA (25.0%), acute gastritis (7.1%), ureter stone (3.6%), constipation (3.6%), and acute appendicitis (3.6%) (Table 2).

**Laboratory and radiologic findings of patients with left primary epiploic appendagitis**

Elevated white blood cell (WBC) count up to 10000/mm<sup>3</sup> was noticed in four of the 26 patients (15.4%). The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were increased in two of the 13 patients (15.4%) and one of the 14 patients (7.1%), respectively. All patients underwent CT scans, and the average size of the PEA was 2.3 ± 0.6 cm (range, 1.0-3.7 cm). It was de-

**Table 3 Computer tomography features of left primary epiploic appendagitis**

Features	Value
Location	
Descending colon	18 (64.3)
Sigmoid-descending junction	3 (10.7)
Sigmoid colon	7 (25.0)
Size (mm)	2.3 ± 0.6
Shape	
Oval	22 (78.6)
Semicircular	4 (14.3)
Triangular	2 (7.1)
Computer tomography features	
Ovoid fatty mass	28 (100.0)
Hyperattenuated ring sign	25 (89.3)
Disproportionate fat stranding	4 (14.3)
Bowel wall thickening±compression	6 (21.4)
Central hyperdense dot/line	5 (17.9)
Lobulated appearance	0 (0.0)

Values are presented as mean ± SD or n (%).

tected most frequently in the descending colon (64.3%), the sigmoid colon (25.0%), and the sigmoid-descending junction (10.7%) in that order. The characteristic CT findings (Figure 1A) were demonstrated in all patients; ovoid fatty mass was found in all patients (100%), hyperattenuated ring sign was detected in 25 patients (89.3%), disproportionate fat stranding was noticed in 4 patients (14.3%), bowel wall thickening with or without compression was observed in 6 patients (21.4%), and central hyperdense dot/line (Figure 1B) was detected in 5 patients (17.9%) (Table 3). We performed follow up CT scans in five patients between 1 and 3 wk, and they showed resolution of the inflammation.

**Treatments and clinical results of patients with left primary epiploic appendagitis**

Surgical management was not required in any of the cases. Twenty-two patients (78.6%) were hospitalized, and the mean length of hospital stay was 5.4 ± 5.0 d (range, 0-24 d). Twenty-two patients (78.6%) received antibiotic therapy, and 6 patients (21.4%) were managed conservatively with hydration and mild analgesics. The duration of antibiotic therapy was 10.5 ± 8.4 d (range, 3-28 d) (Table 4). All patients had clinical follow up except one. No patient experienced symptoms of recurrence within the follow-up period (range, 21 d-105 mo).

**Clinical characteristics, laboratory findings, and treatments of patients with left acute colonic diverticulitis**

During the study period, 25 patients were diagnosed with left ACD. The mean age was 58.8 ± 16.3 years (range, 16-86 years), and 60.0% (15/25) of them were males. The major symptom was sudden onset of localized abdominal pain. Nausea occurred in two patients (8.0%), vomiting in two patients (8.0%), and fever in ten patients (40.0%). The abdominal tenderness was slightly

**Table 4** Treatments and clinical results of patients with left primary epiploic appendagitis and left acute colonic diverticulitis

	Lt. PEA (n = 28)	Lt. ACD (n = 25)	P-value
Hospitalization	22 (78.6)	25 (100.0)	0.014
Treatment			0.474
Antibiotics	22 (78.6)	22 (88.0)	
Conservative management	6 (21.4)	3 (12.0)	
Duration of hospital stay (d)	5.4 ± 5.0	10.2 ± 4.0	< 0.001
Duration of abdominal pain (d)	3.3 ± 2.9	5.6 ± 3.6	0.012
Duration of abdominal tenderness (d)	3.3 ± 1.9	7.2 ± 3.8	< 0.001
Duration of antibiotic therapy (d)	10.5 ± 8.4	16.5 ± 12.6	0.045

Values are presented as mean ± SD or n (%). PEA: Primary epiploic appendagitis; ACD: Acute colonic diverticulitis.

diffuse over the left side of the abdomen (52.0%), and definite rebound tenderness was present in thirteen patients (52.0%) (Table 1). Laboratory tests showed that the WBC, ESR, and CRP were increased in thirteen of the 25 patients (52.0%), nine of the 12 patients (75.0%), and fifteen of the 20 patients (75.0%), respectively. All the patients were hospitalized, and the mean hospitalization period was 10.2 ± 4.0 d (range, 4-20 d). Except for three patients, all were treated with antibiotics (88.0%), and the mean duration of antibiotic therapy was 16.2 ± 12.6 d (range, 6-60 d) (Table 4).

#### Comparison of clinical characteristics and laboratory findings: left primary epiploic appendagitis vs left acute colonic diverticulitis

The mean age was 13.8 years younger in patients with PEA than in ACD (45.0 ± 11.6 years *vs* 58.8 ± 16.3 years, *P* = 0.001). There were no significant differences on sex, body mass index, and underlying disease. All patients showed sudden onset of abdominal pain, but the location of the tenderness was different. In PEA, the pain was more localized (85.7% *vs* 48.0%, *P* = 0.003) in the left lower quadrant (LLQ) area (82.1%), whereas the pain was slightly diffuse throughout the left side of the abdomen in ACD (14.3% *vs* 52.0%, *P* = 0.003). Fever and rebound tenderness were more frequently noted in ACD, and this was statistically significant (7.1% *vs* 40.0%, *P* = 0.004; 14.3% *vs* 52.0%, *P* = 0.003; respectively) (Table 1). WBC, ESR, and CRP were more frequently increased in ACD, which were significantly different from those in PEA (15.4% *vs* 52.0%, *P* = 0.006; 15.4% *vs* 75.0%, *P* = 0.003; 7.1% *vs* 75.0%, *P* < 0.001; respectively).

#### Comparison of treatments and clinical results: left primary epiploic appendagitis vs left acute colonic diverticulitis

The mean duration of hospital stay was about five days shorter (5.4 ± 5.0 d *vs* 10.2 ± 4.0 d, *P* < 0.001), and the mean duration of antibiotic therapy was 6-d shorter (10.5 ± 8.4 d *vs* 16.5 ± 12.6 d, *P* < 0.05) in PEA than in ACD (Table 4). Patients with PEA experienced an improve-

ment of the abdominal pain and tenderness after 3.3 d on average, but in ACD, the abdominal pain and tenderness resolved more than 5 d after treatment. The pain duration was shorter in PEA than in ACD (3.3 ± 2.9 d *vs* 5.6 ± 3.6 d, *P* = 0.012; 3.3 ± 1.9 d *vs* 7.2 ± 3.8 d, *P* < 0.001; respectively) (Table 4).

## DISCUSSION

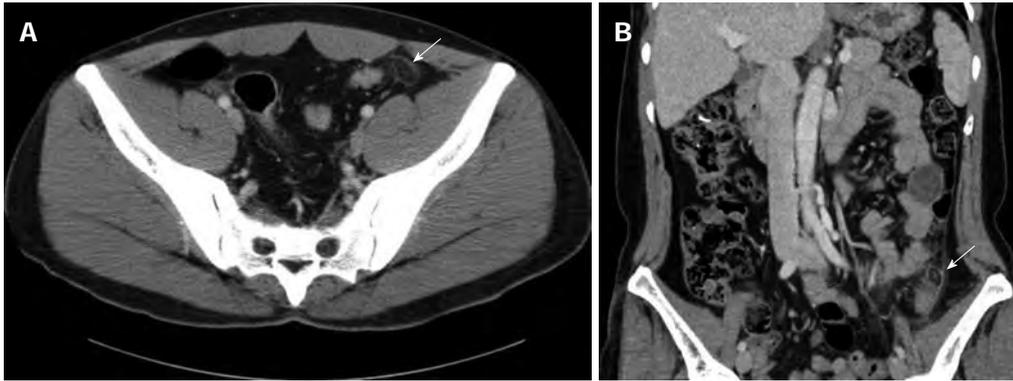
Epiploic appendages, first described in 1543 by Vesalius, are small (1-2 cm thick, 0.5-5.0 cm long) pouches of fat-filled, serosa-covered structures present on the external surface of the colon<sup>[1-4]</sup>. These appendages have not been found to demonstrate any physiologic functions, but are presumed to serve as protective cushions during peristalsis or to provide a defensive mechanism against local inflammation like that of the greater omentum<sup>[2,9,13]</sup>.

PEA, first introduced by Dockerty *et al*<sup>[6]</sup>, is an ischemic inflammatory condition of the epiploic appendages without inflammation of adjacent organs. Each epiploic appendage has one or two small supplying arteries from the colonic vasa recta and has a small draining vein with narrow pedicle<sup>[2,9,14,19,20]</sup>. These appendages are susceptible to torsion due to their pedunculated shape with excessive mobility and limited blood supply<sup>[2,5,9]</sup>. PEA occurs usually from torsion of epiploic appendages which can result in ischemia, or spontaneous venous thrombosis of a draining vein<sup>[5-9]</sup>.

PEA can occur at any age (reported range, 12-82 years<sup>[13]</sup>) with a peak incidence in the fourth to fifth decades, and men are slightly more affected than women<sup>[3,5,7,12,19,20]</sup>. In the current study, the mean age of patients with PEA was 45 years and there was a slight male predominance (16 male *vs* 12 female). They were younger than patients with ACD, and this is consistent with the results of previous studies<sup>[12]</sup>.

Patients with PEA most commonly present with sudden onset of abdominal pain over the affected area, more often in the LLQ mimicking acute sigmoid diverticulitis<sup>[3,15,19,20]</sup>. They usually are afebrile and don't have nausea or vomiting<sup>[2,7,8,19]</sup>. A well-localized abdominal tenderness is present in most patients on physical examination and rebound tenderness is also commonly detected<sup>[8,21]</sup>. A mass may be palpable in 10%-30% of patients<sup>[22]</sup>. In the present study, patients with PEA all showed sudden onset of abdominal pain, and the tenderness was well-localized in the LLQ area. Rebound tenderness was found only in 14.3%, and a palpable mass was noted in 3.6%. In ACD, the patients also had sudden onset of abdominal pain, but the tenderness was diffusely distributed throughout the left side of the abdomen. They more frequently presented with nausea, vomiting, fever, and rebound tenderness, which corresponded well with those of an earlier study<sup>[19]</sup>.

There are no pathognomonic diagnostic laboratory findings in PEA. The WBC and ESR are normal or only moderately elevated<sup>[3,7,8,19]</sup>. In the current study, WBC, ESR, and CRP of the patients with PEA were elevated



**Figure 1** Computer tomography image of a 31-year-old man who presented with acute left lower quadrant pain. A: A 31-year-old man who presented with acute left lower quadrant pain. An oval fatty mass with a hyperattenuated ring and surrounding inflammation adjacent to the sigmoid colon (arrow) is noted. The lesion corresponds to the site of tenderness and is characteristic of primary epiploic appendagitis; B: A 48-year-old female who presented with left lower quadrant pain. An ovoid fat attenuated mass with a central high attenuation area within the inflamed epiploic appendage in the distal descending colon (arrow) is shown.

only in 7%-15% of patients. The patients with ACD more often showed elevations of WBC, ESR, and CRP.

Normal epiploic appendages are usually not identifiable at CT scan without surrounding intraperitoneal fluid such as ascites or hemoperitoneum<sup>[3]</sup>. These appendages typically have fat attenuations, but the attenuation is slightly increased when inflamed<sup>[2,7]</sup>. In the past, PEA has been diagnosed incidentally at laparotomy<sup>[7,8,10]</sup>, but currently it may be possible to make the correct diagnosis with the pathognomonic radiologic findings before operation.

PEA can arise on any segment of the colon. The most frequently involved sites of PEA are the sigmoid colon<sup>[3,15]</sup> and the descending colon followed by the cecum<sup>[15,19]</sup>, where they have more elongated epiploic appendages<sup>[23]</sup>. The characteristic CT finding of PEA is an ovoid fatty lesion with a hyperattenuated ring sign surrounded by inflammatory changes<sup>[9-11,14-16,24]</sup>. A high-attenuated central dot within the inflamed appendage was found in 42.9% by Ng *et al*<sup>[14]</sup>, and in 54% by Singh *et al*<sup>[15]</sup>. It may be due to a thrombosed vessel in the epiploic appendages<sup>[8,10,11,14-16,25]</sup>, or fibrous septa<sup>[26]</sup>. In addition, PEA can appear lobulated when two or more contiguous epiploic appendages lying in close proximity are affected<sup>[8,14,26]</sup>, which would help to differentiate a PEA from an omental infarction<sup>[14]</sup>. In the present study, an ovoid fatty mass with a hyperattenuated ring sign was detected in most PEA patients (89.3%), which is similar to previous studies<sup>[8-10,14,15,23]</sup>. However, we did not find any lobulated appearing PEA on the CT scans.

The common presumptive clinical diagnosis for patients with PEA before radiologic interventions was either diverticulitis or appendicitis. Mollà *et al*<sup>[26]</sup> reported that 7.1% of patients investigated to exclude sigmoid diverticulitis had radiologic findings of PEA. Rao *et al*<sup>[10]</sup> reported that among eleven PEA found on CT scans, seven patients were initially misdiagnosed as having diverticulitis or appendicitis. In the current study, diverticulitis accounted for 57.1% of the presumptive diagnosis in patients with PEA. Only 25.0% of the patients were

suspected of having PEA, most of which were made after the year 2005 when clinicians began to recognize this disease entity.

Early radiologic examination with an abdominal CT scan has aided in the differentiation of PEA from other diseases that require antibiotic therapy or surgical management<sup>[26]</sup>. With the increasing use of CT in the evaluation of an acute abdomen, the incidence of PEA is likely to increase as well<sup>[8,11,12]</sup>. In the present study, only four patients were diagnosed with PEA before 2005, and the rest were diagnosed after 2005.

PEA is a benign and self-limited condition with recovery occurring in less than 10 d without antibiotic therapy or surgery<sup>[7,10,13,23,26]</sup>. In general, patients with PEA can be managed conservatively with oral anti-inflammatory medications<sup>[7,8]</sup>. However, Sand *et al*<sup>[3]</sup> showed 40% recurrence rate in PEA. They believed that conservative treatment may lead to a tendency for recurrence and surgical interventions may be necessary for recurrent cases<sup>[3,12]</sup>. In the current study, most patients (78.6%) with PEA received antibiotic therapy due to the possibility of a more severe diagnosis. No recurrence was noted during the follow-up period, even in cases that were managed conservatively. The follow up CT scan for five patients showed resolution of PEA.

No previous studies were specifically designed to compare the clinical characteristics of patients presenting with left-sided abdominal pain. However, there are some limitations to this study, such as being a relatively small series, retrospective analysis, and no pathologic confirmation of PEA. Further prospective, larger, and comparative studies between left PEA and left ACD are needed.

In conclusion, the clinical symptoms of left PEA and left ACD are very similar in that both types of patients present with left-sided abdominal pain. Although PEA is rare, if a patient has a well-localized abdominal tenderness without associated fever, rebound tenderness, or laboratory abnormalities, we should suspect the diagnosis of PEA and early CT scans should be performed.

PEA can show characteristic CT findings that may allow clinicians to diagnose it correctly and avoid unnecessary hospitalization, antibiotic therapy, or even surgical interventions.

## COMMENTS

### Background

Primary epiploic appendagitis (PEA) is a rare cause of localized abdominal pain. In the past, PEA has been diagnosed incidentally at laparotomy, but currently, it may be possible to make the correct diagnosis with the characteristic radiologic findings before operation. On clinical examination, left PEA can mimic left acute colonic diverticulitis (ACD) owing to the lack of pathognomonic clinical features. No previous studies were specifically designed to compare the clinical characteristics of left PEA with those of left ACD.

### Research frontiers

PEA could be managed conservatively without antibiotic therapy or surgery, but ACD should be treated with antibiotics. Therefore, definitive diagnosis of PEA is important to avoid unnecessary hospitalizations, antibiotic therapy, or even surgical interventions. Early radiological examination with abdominal computer tomography (CT) is useful in obtaining an accurate diagnosis of PEA. The present study was designed to describe the clinical characteristics of left PEA and to compare them with those of left ACD. In addition, authors investigated the characteristic CT findings of PEA.

### Innovations and breakthroughs

The patients with PEA and those with ACD all showed sudden onset of abdominal pain, but the location of the tenderness was different. The patients with PEA had a well-localized abdominal tenderness in the left lower quadrant area, whereas those with ACD presented with slightly diffuse abdominal tenderness throughout the left side of the abdomen. In the ACD cases, fever and rebound tenderness were more often noted, and white blood cell count, erythrocyte sedimentation rate, and C-reactive protein levels were more frequently increased. PEA showed characteristic CT findings like an ovoid fatty lesion with a hyperattenuated ring sign surrounded by inflammatory changes.

### Applications

When patients have well-localized abdominal tenderness without associated systemic manifestation or laboratory abnormalities, clinicians should suspect a diagnosis of PEA and consider performing a CT scan. The data could be useful in obtaining an accurate diagnosis of PEA. The characteristic CT findings and specific clinical features of PEA make it easy to differentiate the disease from diverticulitis.

### Terminology

PEA is an ischemic inflammatory condition of the epiploic appendages without an inflammation of adjacent organs.

### Peer review

The authors demonstrated the clinical features and CT findings of PEA by comparison with those of ACD. The manuscript is well organized and well written.

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## Antiviral therapy delays esophageal variceal bleeding in hepatitis B virus-related cirrhosis

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### Abstract

**AIM:** To investigate the effect of antiviral therapy with nucleoside analogs in hepatitis B virus (HBV)-related cirrhosis and esophageal varices.

**METHODS:** Eligible patients with HBV-related cirrhosis and esophageal varices who consulted two tertiary hospitals in Beijing, China, the Chinese Second Artillery General Hospital and Chinese PLA General Hospital, were enrolled in the study from January 2005 to December 2009. Of 117 patients, 79 received treatment with different nucleoside analogs and 38 served as controls. Bleeding rate, change in variceal grade and non-bleeding duration were analyzed. Multivariate Cox proportional hazard regression was used to identify factors related to esophageal variceal bleeding.

**RESULTS:** The bleeding rate was decreased in the

antiviral group compared to the control group (29.1% vs 65.8%,  $P < 0.001$ ). Antiviral therapy was an independent factor related to esophageal bleeding in multivariate analysis (HR = 11.3,  $P < 0.001$ ). The mean increase in variceal grade per year was lower in the antiviral group ( $1.0 \pm 1.3$  vs  $1.7 \pm 1.2$ ,  $P = 0.003$ ). Non-bleeding duration in the antiviral group was prolonged in the Kaplan-Meier model. Viral load rebound was observed in 3 cases in the lamivudine group and in 1 case in the adefovir group, all of whom experienced bleeding. Entecavir and adefovir resulted in lower bleeding rates (17.2% and 28.6%, respectively) than the control ( $P < 0.001$  and  $P = 0.006$ , respectively), whereas lamivudine (53.3%) did not ( $P = 0.531$ ).

**CONCLUSION:** Antiviral therapy delays the progression of esophageal varices and reduces bleeding risk in HBV-related cirrhosis, however, high-resistance agents tend to be ineffective for long-term treatment.

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**Key words:** Nucleoside analog; Esophageal variceal bleeding; Hepatitis B virus; Cirrhosis; Resistance; Entecavir; Lamivudine; Adefovir

**Core tip:** Antiviral therapy with nucleoside analogs improves clinical outcome in hepatitis B virus (HBV)-related decompensated cirrhosis. However, the emergence of resistance results in liver injury. The consequences may be worse in patients with esophageal varices (EV), in which bleeding and death often occur. This study evaluated the efficacy of antiviral treatment over 5 years in patients with HBV-related cirrhosis and EV and found that antiviral therapy decreased the risk of bleeding. However, agents with a high rate of virological breakthrough were ineffective in preventing bleeding. These findings provide evidence-based suggestions for the treatment of this special group of patients.

Li CZ, Cheng LF, Li QS, Wang ZQ, Yan JH. Antiviral therapy

delays esophageal variceal bleeding in hepatitis B virus-related cirrhosis. *World J Gastroenterol* 2013; 19(40): 6849-6856 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6849.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6849>

## INTRODUCTION

Liver cirrhosis is a common disease that poses a serious threat to the health of patients, and hepatitis B virus (HBV) infection is one of the main causes of cirrhosis. The 5-year survival rate of decompensated liver cirrhosis is reported to be only 14%-28%<sup>[1,2]</sup>. Nucleoside analogs, by inhibiting HBV polymerase and decreasing HBV load, have been widely used in the treatment of hepatitis B. Antiviral therapy with sustained suppression of viral replication has shown benefits in decompensated cirrhotic patients. However, there are no detailed reports of the effects of antiviral therapy in esophageal variceal bleeding, one of the most dangerous and life-threatening complications of cirrhosis.

About 30% of patients with cirrhosis and esophageal varices (EV) will experience bleeding during their lifetime<sup>[3]</sup>. A single episode of uncontrolled variceal bleeding results in immediate death in 5%-8% of patients and has a six-week mortality rate of at least 20%<sup>[4]</sup>. Therefore, prevention of variceal bleeding is of paramount importance.

A number of studies have shown that antiviral therapy with nucleoside analogs is associated with improved clinical outcome in patients with HBV-related decompensated cirrhosis<sup>[5,6]</sup>. However, the problem of drug resistance has also emerged with the long-term use of these agents<sup>[7,8]</sup>. The emergence of resistance results in a loss of virological suppression, which in turn causes progressive liver injury and even severe liver failure<sup>[9]</sup>. The consequences may be worse in patients with EV. Virological breakthrough in patients with EV usually results in EV bleeding or even death.

A retrospective study by Koga *et al*<sup>[10]</sup> demonstrated an improvement in patients with esophageal varices following lamivudine (LAM) treatment. However, the study consisted of only 12 patients treated with LAM and 6 controls, and there were no data on bleeding rate and the effect of virological breakthrough. As patients usually experience bleeding when virological breakthrough takes place, it is necessary to separate the patients and evaluate the harm of drug resistance and the benefit of HBV suppression in these patients to provide evidence-based treatment suggestions. The aim of the present study was to evaluate the efficacy of antiviral treatment over 5 years in patients with HBV-related cirrhosis and EV.

## MATERIALS AND METHODS

### Study design

This study evaluated the efficacy of antiviral therapy with different nucleoside analogs in patients with HBV-related

cirrhosis and EV. Eligible patients with HBV-related cirrhosis who consulted two tertiary hospitals in Beijing, China, the Chinese Second Artillery General Hospital and Chinese PLA General Hospital, were enrolled in the study from January 2005 to December 2009.

The study population had HBV-related cirrhosis and EV. All patients had serum HBV DNA > 500 copies/mL as measured by polymerase chain reaction (PCR) (Fluorescence Quantitation kit; Shanghai Kehua Bio-engineering, Shanghai, China). Exclusion criteria included: history of hepatitis C or D, viral hepatitis, evidence of alcoholic cirrhosis, history of surgery for portal hypertension (splenectomy, shunt or devascularization), suspected liver cancer, hepatic encephalopathy or hepatorenal syndrome, or life-threatening diseases.

In accordance with clinical practice guidelines<sup>[11-13]</sup>, endoscopic eradication of varices was performed in cases with a history of bleeding. Other treatment modalities, such as polyene phosphatidylcholine, reduced glutathione and diuretics, were administered as required. Propranolol was prescribed to all patients except for those who could not tolerate, or had contraindications to, beta-blocker use.

Because no evidence-based suggestions on antiviral therapy in patients with esophageal varices were available, the benefit and potential harm (such as drug resistance and adverse effects) of antiviral therapy were introduced to the patients. Patients received one of the following antiviral treatments immediately after enrollment: lamivudine (LAM) (100 mg/d, Glaxo Wellcome, United Kingdom), adefovir (ADV) (10 mg/d, Tianjin Pharmaceutical, China), entecavir (ETV) (0.5 mg/d, Bristol-Myers Squibb, United States), telbivudine (LdT) (600 mg/d, Novartis, Switzerland), a combination of LAM and ADV (LAM 100 mg/d + ADV 10 mg/d), or no antiviral therapy according to their wishes. As antiviral therapy may have a greater risk in patients with esophageal varices, it was not prescribed if the patients refused it for reasons such as adverse effects and resistance.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of both Hospitals. All patients provided written informed consent prior to enrollment in the study.

### Efficacy assessment

Patients were followed up at a 3-mo interval over 5 years, and levels of HBV DNA, glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), total bilirubin, serum albumin, prothrombin time, platelet count, diameter of portal vein and splenic area on ultrasound scan were recorded.

Endoscopic findings were graded according to the criteria of the Japanese Association of Portal Hypertension<sup>[14]</sup> as Grade I, II or III, and given a score of 1, 2 or 3, respectively. Bleeding was scored as 4. Patients whose varices were eradicated were scored as 0. Those who showed newly visible, small vessels after endoscopic

**Table 1** Demography of the patients (mean  $\pm$  SD)

	Non- antiviral ( <i>n</i> = 38)	Antiviral ( <i>n</i> = 79)	<i>P</i> value	Normal range
Gender (male/female)	30/8	62/17	1.000	
Age (yr)	51.8 $\pm$ 10.3	50.4 $\pm$ 8.8	0.443	
Median Child-Pugh score	8	8	0.887	
Previous grade of EV (0/1/2/3)	21/0/6/11	41/4/18/16	0.330	
Previous copies of HBVDNA (log10)	4.2 $\pm$ 1.1	4.6 $\pm$ 2.5	0.176	(-)
Previous hepatitis B e antigen (+/-)	24/14	46/33	0.689	(-)
Previous diameter of portal vein (mm)	11.8 $\pm$ 2.1	12.6 $\pm$ 2.2	0.392	< 10
Previous splenic section area (cm <sup>2</sup> )	35.3 $\pm$ 9.5	37.4 $\pm$ 13.2	0.384	< 20
GPT (IU/L)	49.7 $\pm$ 21.1	53.3 $\pm$ 64.9	0.315	5-40
GOT (IU/L)	48.1 $\pm$ 32.8	57.9 $\pm$ 56.2	0.231	5-40
TB (mmol/L)	26.7 $\pm$ 14.6	25.1 $\pm$ 15.5	0.635	3.4-17.1
ALB (g/L)	24.7 $\pm$ 4.9	25.4 $\pm$ 5.0	0.667	35-50
PT (s)	14.9 $\pm$ 2.5	16.5 $\pm$ 7.9	0.236	< 12
PLT (10 <sup>12</sup> /L)	83.1 $\pm$ 41.8	78.7 $\pm$ 41.9	0.600	100-300
Endoscopic eradication (yes/no)	17/21	37/42	0.846	
Propranolol (yes/no)	30/8	65/14	0.801	

EV: Esophageal varices; HBV DNA: Hepatitis B virus DNA; GPT: Glutamic-pyruvic transaminase; GOT: Glutamic-oxaloacetic transaminase; TB: Total bilirubin; ALB: Serum albumin; PT: Prothrombin time; PLT: Platelet count.

eradication, but did not reach grade I were scored as 0.5. Patients without a history of bleeding underwent endoscopic examination every 2 follow-up visits (at a 6-mo interval). Cases with a history of bleeding underwent endoscopic eradication of the varices and follow-up at month 3, and then at a 6-mo interval over the remaining study period. Variceal scores were recorded.

The end-point of this observational study was bleeding or time to study conclusion (December 2009) if the patient did not experience any bleeding. The duration from study enrollment to bleeding or to study conclusion was defined as non-bleeding duration.

### Safety

The incidence of adverse events, discontinuations and deaths were documented. Complete blood counts, blood urea nitrogen, creatinine, creatine kinase, lactic acid and electrolytes were also monitored.

### Statistical analysis

The software STATA 10.0 (Stata Corporation, United States) was used in the statistical analysis of data. The bleeding rates (defined as the cumulative proportion of patients who experienced an esophageal variceal bleeding episode during the study) in the antiviral and control groups were compared using the  $\chi^2$  test. Multivariate Cox proportional hazard regression was used to identify factors related to esophageal variceal bleeding. Non-bleeding duration of the different groups was determined using the Kaplan-Meier model. Increases in variceal score were compared between groups using the Student's *t* test. The portal vein diameter and splenic area on ultrasound scan, levels of GPT, GOT, total bilirubin, serum albumin, prothrombin time and platelet counts were also compared using the Student's *t* test. All tests were two-sided and a *P* value of < 0.05 was considered statistically significant.

## RESULTS

### Patient characteristics

A total of 117 patients who fulfilled the study criteria were enrolled from January 2005 to December 2009. Of these, 79 patients received antiviral therapy, including ETV (*n* = 29), ADV (*n* = 28), LAM (*n* = 15), LdT (*n* = 4) and combination treatment with LAM and ADV (*n* = 3). Patients not receiving antiviral therapy (*n* = 38) were followed up as a control group, and received the same treatment as the antiviral group minus antiviral therapy. Of the total study population, 63 patients (42 in the antiviral group and 21 in the control group) who had a history of bleeding underwent endoscopic eradication of varices, while the other 54 cases (37 in the antiviral group and 17 in the control group) without a history of esophageal variceal bleeding did not undergo endoscopic eradication treatment. Primary prevention was not performed in this study population. In addition, a total of 95 patients (65 patients in the antiviral group and 30 patients in the control group) received propranolol. Both propranolol and endoscopic eradication patterns were not significantly different between the two groups (*P* = 0.801 and *P* = 0.846, respectively). The antiviral treatment and control groups were well matched with regard to gender, age, hepatic function, grade of EV, HBV DNA level, HBeAg status, portal vein diameter and splenic section area on ultrasound (Table 1).

### Virological response

By week 12 of treatment, HBV DNA decreased to undetectable levels (defined as < 500 copies/mL, by PCR) in 58 (73.4%) of the 79 patients in antiviral treatment group. This was observed in 23 (79.3%) patients in the ETV group, 19 (67.8%) patients in the ADV group, 12 (80.0%) patients in the LAM group, 2 patients in the Ldt group (*n* = 4) and 2 patients in the LAM/ADV treatment group (*n* = 3). The number of patients achieving undetectable

**Table 2** Laboratory examination results, portal diameter and splenic area in antiviral and non-antiviral groups during the study (mean  $\pm$  SD)

	Non-antiviral ( <i>n</i> = 38)	Antiviral ( <i>n</i> = 79)	<i>P</i> value
GPT (IU/L)	19.2 $\pm$ 53.1	-28.6 $\pm$ 58.8	< 0.001
GOT (IU/L)	19.9 $\pm$ 52.8	-34.9 $\pm$ 86.2	0.001
TB (mmol/L)	3.9 $\pm$ 9.6	-3.8 $\pm$ 9.5	0.009
ALB (g/L)	-2.8 $\pm$ 5.5	1.2 $\pm$ 6.1	0.003
PT (s)	1.4 $\pm$ 2.2	-0.2 $\pm$ 2.1	0.001
PLT ( $10^{12}$ /L)	-18.9 $\pm$ 28.8	-10.3 $\pm$ 16.9	0.029
Diameter of portal vein (mm)	1.0 $\pm$ 0.9	0.2 $\pm$ 0.8	< 0.001
Splenic section area (cm <sup>2</sup> )	8.6 $\pm$ 7.2	3.6 $\pm$ 9.8	0.012

GPT: Glutamic-pyruvic transaminase; GOT: Glutamic-oxaloacetic transaminase; TB: Total bilirubin; ALB: Serum albumin; PT: Prothrombin time; PLT: Platelet count.

HBV DNA levels increased to 67 at week 24. At week 48, a total of 71 patients in the antiviral group achieved undetectable HBV DNA levels. None of the patients in the control group (*n* = 38) achieved undetectable HBV DNA during the study.

#### Reduced deterioration of cirrhosis due to antiviral therapy

The portal vein diameter and area of splenic section tended to increase at the conclusion of the study compared to baseline. However, there was a smaller mean increase in the diameter of the portal vein and area of splenic section on ultrasound in the antiviral group compared to the control group (Table 2). Analysis of biochemical profiles (alanine aminotransferase, aspartate aminotransferase, serum albumin, total bilirubin and prothrombin time) showed progressive improvements in liver function in the group receiving antiviral treatment (Table 2). Median Child-Pugh score increased from 8 to 10 in control patients (*P* = 0.018), and decreased from 8 to 7 in the antiviral group (*P* = 0.686).

#### Reduced bleeding rate and delayed progression of variceal grade due to antiviral therapy

By the end of the study, the bleeding rate was significantly decreased in the antiviral group (*n* = 79) compared to the control group (*n* = 38) (29.1% vs 65.8%, *P* < 0.001). The bleeding rate was also statistically different when all the patients were stratified into the endoscopic eradication group and non-endoscopic intervention group (Table 3). The mean  $\pm$  SD increase in variceal score was reduced in the antiviral treatment group compared with the control group (Table 3).

Multivariate Cox regression analysis was performed to identify the factors associated with increased or decreased esophageal bleeding. The results showed that antiviral therapy (OR = 11.3, 95%CI: 3.1-38.5; *P* < 0.001), endoscopic eradication of varices (OR = 15.8, 95%CI: 4.1-51.1; *P* < 0.001), baseline portal vein diameter (OR = 39.1, 95%CI: 1.6-842.6; *P* = 0.025) and baseline serum HBV DNA level (OR = 0.8, 95%CI: 0.6-1.1; *P* = 0.042)

**Table 3** Increase in score of varices/year in antiviral and non-antiviral therapy

	Non-antiviral	Antiviral	<i>P</i> value
All the cases ( <i>n</i> = 117)	1.7 $\pm$ 1.2	1.0 $\pm$ 1.3	0.003
Endoscopic eradication ( <i>n</i> = 63)	1.8 $\pm$ 1.5	1.1 $\pm$ 1.6	0.098
No endoscopic intervention ( <i>n</i> = 54)	1.6 $\pm$ 0.7	0.8 $\pm$ 0.9	0.003

were independent factors related to esophageal bleeding.

#### Virological breakthrough hindered the benefit of antiviral therapy

Four cases showed undetectable HBV DNA initially, however, viral load rebound was observed during follow-up, which was defined as virological breakthrough. All 4 cases bled in contrast to other patients who benefited from antiviral therapy.

In the LAM group (*n* = 15), three cases were found to have virological breakthrough during the study. All 3 cases had no history of bleeding and therefore were not treated with endoscopy. In the first case, virological breakthrough was detected at week 48 ( $1.8 \times 10^5$  copies/mL) and ADV was administered immediately, however, the patient exhibited re-bleeding at week 53 and the HBV DNA level reached  $3.3 \times 10^5$  copies/mL. In the second case, the patient bled at week 192 and eventually died due to bleeding. Examination revealed HBV DNA at  $5.1 \times 10^5$  copies/mL. In the third case, the patient bled at week 90 and HBV DNA breakthrough was detected on examination ( $3.1 \times 10^8$  copies/mL). The patient was treated with vasoactive drugs, endoscopic eradication of varices and switched to ETV after cessation of bleeding. HBV DNA levels were subsequently undetectable at 12 wk after treatment, and the patient was bleed-free for 48 wk by the end of the study.

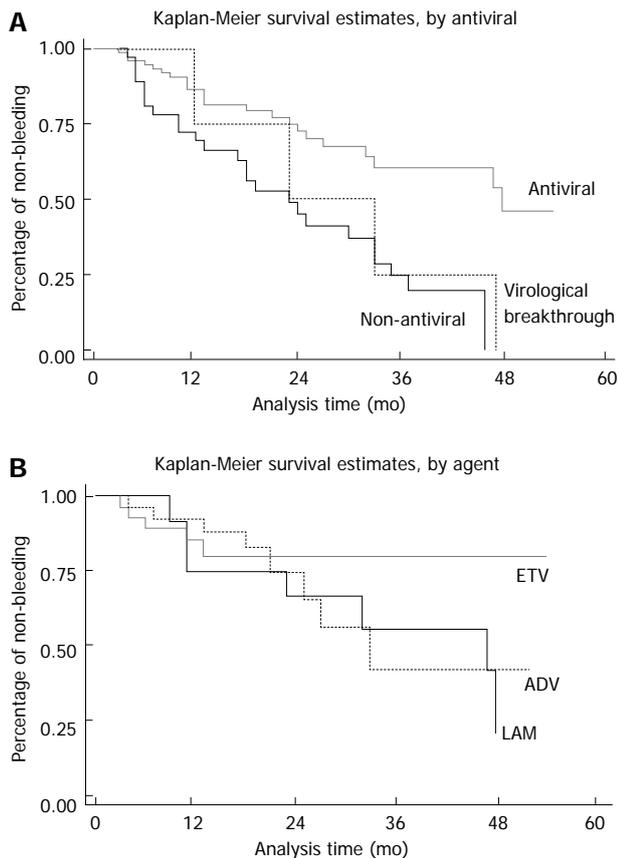
One patient in the ADV group (without a history of bleeding and endoscopic intervention) experienced bleeding at week 130. HBV DNA was  $9.8 \times 10^3$  copies/mL at examination. The patient was switched to ETV after cessation of bleeding and was bleed-free for 36 wk by the end of the study.

As more cases suffered from virological breakthrough and bleeding, the bleeding rate in the LAM group was not statistically different from that in the control group (8/15 vs 25/38, *P* = 0.531), while the ETV (5/29) and ADV (8/28) groups showed a lower bleeding rate (*P* < 0.001 and *P* = 0.006, respectively) compared to the control group. ETV was statistically better than LAM (*P* = 0.019), and no significant differences were found between the other agents.

There was one case of bleeding in both the LdT (*n* = 4) and combination treatment (LAM/ADV) groups (*n* = 3), and due to the small number of cases, they were not included in the statistical analysis.

#### Kaplan-Meier analyses of non-bleeding duration

Kaplan-Meier analyses demonstrated that bleeding was



**Figure 1** Kaplan-Meier analysis of non-bleeding duration. A: Non-bleeding duration in antiviral and control cases was compared using the Kaplan-Meier survival model. Bleeding was defined as a failed event. The occurrence of bleeding was reduced and delayed in the antiviral treatment group compared to the control group. The curve of virological breakthrough cases (dashed line) was close to that of the control group, demonstrating reduced efficacy when virological breakthrough occurred; B: Non-bleeding durations for entecavir (ETV), adefovir (ADV) and lamivudine (LAM) therapy were compared using the Kaplan-Meier survival model. Bleeding was defined as a failed event. The non-bleeding durations for ETV, ADV and LAM were similar in the first 2 years, however, differences became clear following long-term treatment, which may have been due to cumulative resistance and bleeding.

postponed in the antiviral treatment group compared to the control group (Figure 1A). However, the curve of virological breakthrough cases was close to that of the control group (Figure 1A). The Kaplan-Meier curves of ETV, ADV and LAM crossed each other within the first 2 years, but deviated beyond the 2-year mark (Figure 1B).

### Safety

One patient died of esophageal variceal bleeding as stated above; no other severe adverse events were reported in the study. Most reported side effects were mild such as fatigue ( $n = 1$ ), headache ( $n = 1$ ), dyspepsia ( $n = 2$ ), nausea ( $n = 1$ ), dizziness ( $n = 1$ ) and insomnia ( $n = 1$ ). Discontinuation of treatment due to adverse events was not observed.

### Overall survival

In this study, follow-up ended if the patient bled. Additional follow-up was included to determine the effect

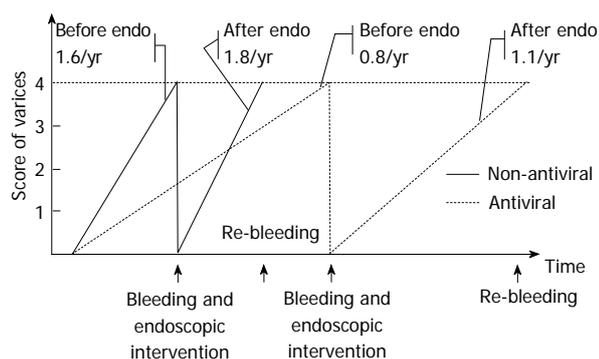
of antiviral therapy on patient survival. The cumulative 1-year survival rate was 97.5% (77/79) and 89.5% (34/38) for the antiviral group and control group, respectively ( $P = 0.086$ ). The cumulative 2-year survival rate was 93.7% (74/79) and 86.3% (29/38) for the antiviral group and control group, respectively ( $P = 0.012$ ). However, 8 patients in the control group switched to antiviral therapy. When these 8 patients were excluded, the cumulative 2-year survival rate in the control group was 70.0% (21/30), which was also lower than in the antiviral group ( $P = 0.002$ ). Most of the patients in the control group switched to antiviral therapy during the additional follow-up period, and a comparison of the 5-year survival rate of the control group and antiviral group was not available. In addition to the patient who died of drug resistance and bleeding, 17 patients died (9 due to bleeding, 3 due to hepatic encephalopathy, 2 due to chronic liver failure and 3 due to liver cancer) and 17 were lost during the additional follow-up in the antiviral group. The overall 5-year survival rate in the antiviral group was 71.0%, which was higher than in the general cirrhotic patients<sup>[1]</sup>.

## DISCUSSION

Esophageal variceal bleeding is a life-threatening complication of decompensated cirrhosis, and bleeding from varices is a medical emergency which requires immediate treatment. If bleeding is not controlled quickly, the patient may go into shock or die. Aside from the urgent need to stop the bleeding, treatment is also aimed at the prevention of future bleeding. Treating the underlying cause of variceal bleeding can help prevent recurrence and early treatment of liver disease may prevent the development of varices.

Propranolol is accepted as the main treatment modality for the prevention of esophageal variceal bleeding in cirrhotic patients<sup>[15,16]</sup>. Preventive endoscopic intervention is advocated by some medical experts in high-risk cases. For patients who have experienced bleeding, the accepted modalities for the prevention of re-bleeding include endoscopic eradication of varices (secondary prophylaxis)<sup>[11-13]</sup>, continuous administration of propranolol and regular endoscopic follow-up plus supplementary endoscopic intervention<sup>[17-19]</sup>. The present study examined the role of antiviral therapy as an additional option for the prevention of esophageal variceal bleeding in hepatitis B patients.

Nucleoside analog treatment in decompensated cirrhosis has been widely accepted in recent years<sup>[20-22]</sup>, however, there have been no studies on the efficacy of antiviral treatment in patients with both cirrhosis and esophageal varices. In this study, antiviral therapy delayed progression of EV and decreased bleeding rates in cirrhotic patients with actively replicating HBV. Figure 2 shows the model of varices development. The grade of EV increases with time resulting in bleeding. After endoscopic eradication, the variceal score increases again until bleeding recurs. Antiviral therapy delayed both the progression of varices before and after endoscopic therapy



**Figure 2** Sketch of delayed progression of esophageal varices by antiviral therapy. Varices were scored according to the criteria of the Japanese Association of Portal Hypertension: grade I, II, and III and given a score of 1, 2 and 3, respectively. Bleeding was scored as 4. Endoscopic eradication was scored as 0. The dark line shows the development of esophageal varices in hepatitis B virus-related cirrhosis without antiviral therapy. The dashed line is the development of varices with antiviral therapy. The grade of esophageal varices increases with time and then bleeding occurs. After endoscopic eradication, the varices score increases again until bleeding recurs. Antiviral therapy delayed both the progression of varices before and after endoscopic therapy and postponed esophageal variceal bleeding. Before endo: Before endoscopic intervention; After endo: After endoscopic intervention.

and postponed esophageal variceal bleeding (*i.e.*, effective in both primary and secondary prophylaxis).

The delayed progression of EV and decreased bleeding rates in patients receiving antiviral therapy may be explained by relief of portal hypertension. Decreased inflammation due to antiviral therapy might contribute to lower pressure of hepatic sinusoids. In addition, it has been reported that long-term antiviral therapy can lead to histological improvement of cirrhosis, slowing the rate of deterioration<sup>[23]</sup>. Although biopsy of the liver was not performed in this study, the improvement in liver structure was reflected by the delayed dilation of portal veins, delayed increase in spleen size, the reversion of levels of GPT, GOT, bilirubin, prothrombin time, albumin and delayed decrease in platelets count. These clinical benefits, together with delayed bleeding, are the ultimate aims in clinical practice. Recently, an on-line published study reported that ETV therapy reduced the risks of hepatic events in hepatitis B cirrhosis patients within 5 years<sup>[24]</sup>. The results of our study are in accordance with the findings of this published study.

However, drug-resistance was found to be the main obstacle in the benefit of antiviral therapy for the prevention of EV bleeding. All 3 LAM-resistant cases and 1 ADV-resistant case experienced bleeding. As a result, the efficacy of LAM in the prevention of EV bleeding decreased to a level that was not statistically different from that of the control group in the present study. The Kaplan-Meier curves of ETV, ADV and LAM crossed each other within the first 2 years, but deviated beyond the 2-year mark. It is known that drug resistance increases with time. A plausible explanation for this is that the high incidence of virological breakthrough observed with LAM reduced the efficacy of LAM in the preven-

tion of esophageal bleeding. As studies have reported a lower rate of resistance for ETV<sup>[25]</sup>, this agent may be a better choice in the prevention of esophageal bleeding in cirrhotic patients with active viral hepatitis B replication. The LAM/ADV combination has also been reported to have lower drug resistance rates<sup>[26]</sup>. However, combination therapy may be more expensive and result in more adverse events, and therefore, may not be well received by patients.

One limitation of the present study is that genetic screening for emergence of antiviral resistance was not performed. This is an important issue as current treatment guidelines recommend long-term treatment of patients with cirrhosis. Consequently, it is unknown whether the patients who experienced virological breakthrough also had emergence of genotypic resistance. In this study, the levels of HBV DNA, GOT, GPT and other biochemical parameters were tested in all patients at a 3-mo interval, however, only one case was found to have developed virological break through during routine examination. Although rescue treatment with additional ADV was administered immediately, the patient still experienced esophageal bleeding after a few weeks. In the other three patients who experienced bleeding, virological breakthrough had not been previously detected. As all four cases with virological breakthrough were compliant with the antiviral treatment, the most likely explanation for virological breakthrough is antiviral resistance. Investigations of the underlying gene mutations and switching to a more appropriate treatment regimen as early as possible may be useful in enhancing the effectiveness of antiviral therapy.

In conclusion, antiviral therapy with nucleoside analogs may delay progression of varices, decrease the risk of bleeding and improve liver function in patients with HBV-related cirrhosis and EV. However, drug resistance usually leads to bleeding in this special group of patients. Agents with a high rate of virological breakthrough may not be effective in the long-term prevention of esophageal variceal bleeding. The major limitation of the present study is the relatively small sample size of heterogeneous use of antiviral agents. These conclusions should be confirmed in further randomized controlled clinical studies.

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## COMMENTS

### Background

Antiviral therapy with nucleoside analogs improves the clinical outcome of hepatitis B virus (HBV) infection. However, the emergence of resistance results in liver injury. The consequences may be worse in patients with esophageal varices (EV), in which bleeding and death often occur.

### Research frontiers

Recently, an on-line published study demonstrated that entecavir therapy re-

duced the risks of hepatic events in hepatitis B cirrhosis patients within 5 years. Koga *et al* reported an improvement in esophageal varices in patients following lamivudine (LAM) treatment in a retrospective study. However, the study only consisted of 12 patients treated with LAM and 6 controls, and there were no data on bleeding rate and the effect of virological breakthrough. As patients usually experience bleeding when virological breakthrough takes place, it is necessary to separate the patients and evaluate the harm of drug resistance and the benefit of HBV suppression in these patients to provide evidence-based treatment suggestions.

### Innovations and breakthroughs

The present study evaluated the efficacy of antiviral treatment over 5 years in patients with HBV-related cirrhosis and EV, and found that antiviral therapy decreased the risk of bleeding; and agents with a high rate of virological breakthrough were ineffective in preventing bleeding. These findings for the first time provide evidence-based treatment suggestions for this special group of patients.

### Applications

Antiviral therapy with nucleoside analogs may delay progression of varices, decrease the risk of bleeding and improve liver function in patients with HBV-related cirrhosis and EV. Agents with a high rate of virological breakthrough may not be effective in the long-term prevention of esophageal variceal bleeding.

### Terminology

Nucleoside analogs, are molecules that can inhibit HBV polymerase, and therefore decrease HBV load. They have been widely used in the treatment of hepatitis B. Virological breakthrough indicates that the viral load decreased to an undetectable level initially, and rebounded during follow-up. Virological breakthrough often leads to progressive liver injury and even severe liver failure.

### Peer review

The article provides statistical analysis of data from HBV patients who went through antiviral therapy to determine the role of antiviral therapy in esophageal varices and bleeding in HBV-related cirrhosis. This article provides information important to clinicians and HBV patients in general.

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## Trans-umbilical endoscopic cholecystectomy with a water-jet hybrid-knife: A pilot animal study

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### Abstract

**AIM:** To investigate the feasibility and safety of Natural orifice trans-umbilical endoscopic cholecystectomy with a water-jet hybrid-knife in a non-survival porcine model.

**METHODS:** Pure natural orifice transluminal endoscopic surgery (NOTES) cholecystectomy was performed on three non-survival pigs, by transumbilical approach, using a water-jet hybrid-knife. Under general anesthesia, the following steps detailed the procedure: (1) incision of the umbilicus followed by the passage of a double-channel flexible endoscope through an overtube into the peritoneal cavity; (2) establishment of pneumoperitoneum; (3) abdominal exploration; (4) endoscopic cholecystectomy: dissection of the gallbladder performed using water jet equipment, ligation of the cystic artery and duct conducted using nylon loops; and (5) necropsy with macroscopic evaluation.

**RESULTS:** Transumbilical endoscopic cholecystectomy was successfully completed in the first and third pig, with minor bleedings. The dissection times were 137 and 42 min, respectively. The total operation times were 167 and 69 min, respectively. And the lengths of resected specimen were 6.5 and 6.1 cm, respectively. Instillation of the fluid into the gallbladder bed produced edematous, distended tissue making separation safe and easy. Reliable ligation using double nylon loops insured the safety of cutting between the loops. There were no intraoperative complications or hemodynamic instability. Uncontrolled intraoperative bleeding occurred in the second case, leading to the operation failure.

**CONCLUSION:** Pure NOTES trans-umbilical cholecystectomy with a water-jet hybrid-knife appears to be feasible and safe. Further investigation of this technique with long-term follow-up in animals is needed to confirm the preliminary observation.

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**Key words:** Natural orifice transluminal endoscopic surgery; Cholecystectomy; Water-jet; Hybrid-knife; Triangulation

**Core tip:** Flexible single-incision surgery (FSIS), one of recent advances in endoscopic surgery, is a promising single-incision approach, which has exploited the advantages of single-incision laparoscopy and narrow sense of natural orifice transluminal endoscopic surgery (NOTES). Compared to NOTES, FSIS uses the navel to facilitate the extraction of the specimen and the umbilical closure is quick and easy. Compared to SILS, FSIS does not need any specialized device for the entry ports. Furthermore, water-jet hybrid knife technology enables a quick switch between blunt and sharp dissection. This study assessed the feasibility and safety of endoscopic cholecystectomy using transumbilical approach and water jet hybrid knife technology.

Jiang SJ, Shi H, Swar G, Wang HX, Liu XJ, Wang YG. Trans-umbilical endoscopic cholecystectomy with a water-jet hybrid-knife: A pilot animal study. *World J Gastroenterol* 2013; 19(40): 6857-6862 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6857.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6857>

## INTRODUCTION

Laparoscopic cholecystectomy (LC)<sup>[1]</sup> remains the gold standard for the treatment of benign gallbladder diseases, showing superiority such as better cosmetic results, less postoperative pain, and shorter recovery time compared with open cholecystectomy. Under the guidance of the Holy Grail of minimal invasive surgery, namely, scarless surgery, natural orifice transluminal endoscopic surgery (NOTES) has made substantial progress. However, it is also technically difficult due to lack of appropriate devices and loss of triangulation. To achieve both minimal invasiveness and effectiveness, we proposed the following technique for pure NOTES cholecystectomy, by transumbilical approach, using an endoscopic water jet system applied to perform blunt and sharp dissection without accessory switch.

## MATERIALS AND METHODS

### Animals

Natural Orifice Trans-umbilical Cholecystectomy with a new water-jet dissector Erbejet 2 was performed on three non-survival TiBet experimental pigs (two were female weighing 30 kg, the other was male weighing 35 kg).

### Experimental site

Experimental site: Olympus training center for animal experimental study in Peking, China.

### Animal preparation and post-operative management

Animal preparation and post-operative management: fasting 48 h pre-operation and took polyethylene glycol 4 g/kg orally for intestinal preparation before procedure. Preoperative intramuscular ketamine 20 mg/kg and Xylazine 2 mg/kg mixture induced anesthesia, retaining animal on the operating table in a supine position and performing endotracheal intubation, connecting anaesthesia apparatus following inhalational anesthesia by 4%-5% isoflurane. When the animals entered into complete general anesthesia condition, regulating gas concentration to 1%-2% for anesthesia maintenance and intraoperative intravenous management with 5% glucose-saline. After the operation ended, intravenous pentobarbital sodium 100 mg/kg was injected to the porcine, along with the autopsy to observe the wound and adjacent organs for any damages.

### Instruments and devices

Instruments and devices: forward-looking double channel

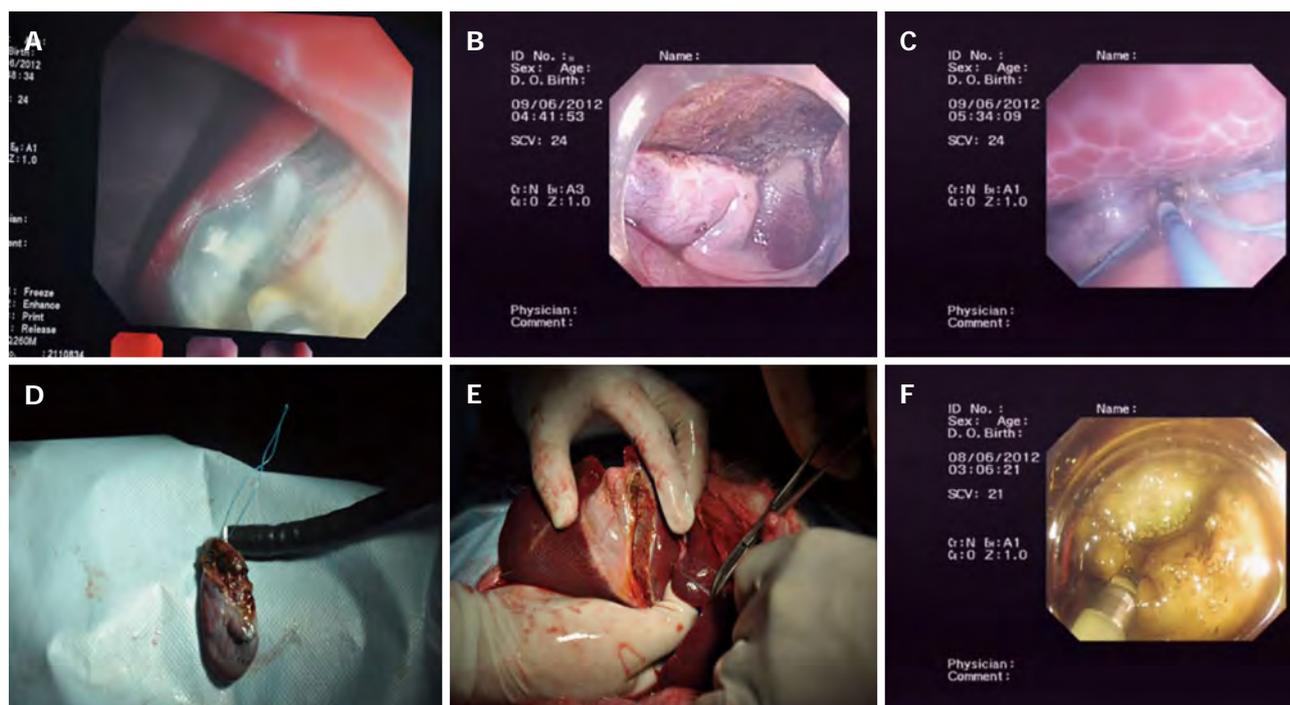
gastroscope(GIF-2TQ260M, olympus), non-invasive forceps (FQ-46L-1, olympus), thermal hemostatic forceps (endoscopic hemostatic forceps FD-410LR, olympus), nylon cord snare (MAJ-340, olympus) and ligation device (HX-20L-1, olympus), overtube (overtubeMD48618, Sumitomo Bakelite, Tokyo Japan), Trocar, co2 air pump, digestive endoscopic surgery workstation (ERBE VIO-300D, Germany), T-type Erbejet2 (ERBEjet2; ERBE Elektromedizin, Germany), endoscopic clips and trip gear (endoclips, HX-610-135OLYMPUS, olympus), methylene blue, normal saline.

### Experimental procedure

**Establishment of pneumoperitoneum:** Porcines were placed under supine position, routine preoperative skin disinfection, spread towel, making a 1.5 cm longitudinal incision through the umbilicus, puncturing into abdominal cavity with trocar then overtube was inserted. Surrounding skin was clamped by using towel clamp in order to avoid air leakage, so endoscope can be inserted into peritoneal cavity through overtube, (being unable to gear co2 air pump directly), the latter is connected to endoscopic air supply device to maintain pneumoperitoneum which can be adjusted by endoscopic inspiration button.

**Abdominal exploration:** After the establishment of pneumoperitoneum, exploration of intra-abdominal viscera from epigastrium to hypogastrium was done. Stomach, diaphragm, gallbladder, spleen, small intestine and large intestine can be viewed meanwhile retroperitoneal organs kidney, pancreas for instance cannot be explored. A swollen light blue gallbladder can be seen from endoscope due to abrasion. Sometimes gallbladder is covered by the lobes of liver that requires removing the lobes of liver to reveal cholecyst by operating endoscope.

**Cholecystectomy:** The assistant manipulated overtube to advance the endoscope to the surgery field and to reveal cholecyst and liver bed. High-pressure injection of methylene blue solution dyed normal saline by using T-type ERBEjet2 through endoscopic work channel into fibrous tissue between gallbladder and liver bed. The water-injection pressure was set to 30-50 bar. Blue cushion formation taking place following the local injection (Figure 1A), demarcates the liver parenchyma. Then the dissection of fibrous tissue was done between gallbladder's serous membrane and liver parenchyma by T-type ERBEjet2 (coagulation-cut hybrid model, power 45w, effect 2) starting from fundus or neck of gallbladder from left to right side. Every separation was done following the cushion formation and was operated in the raised or lift cushion. Once catching the sight of small blood vessels or encountering haemorrhage, prophylactic hemostasis with electro-tome or hemostatic forceps would be required. The gallbladder would fall off liver bed while dissected carefully up to the neck or fundus of gallbladder (Figure 1B). Dissected clearly and completely to reveal calot's triangle and cystic duct, cystic artery, hepatic duct and common bile duct has been identified. Ligation of



**Figure 1** Picture demonstrating a concept of a novel method of cholecystectomy. A: Methylene blue mixed with normal saline solution injected into fibrous tissues between gallbladder and liver bed; B: The gallbladder falling off liver bed; C: Cystic duct and artery cut using the water-jet hybrid-knife between loops; D: The resected gallbladder grasped by forceps; E: Macroscopic view of the liver fossa on necropsy; F: Clipped gallbladder perforation.

cystic duct and cystic artery with nylon cord snare instead of endoclips for fast ligation. During the procedure, non-invasive forceps and ligation device were introduced into abdominal cavity through endoscopic double channels respectively. Cholecyst was clamped and empocketed into nylon cord snare and then ligation of the cystic duct and cystic artery together was done on the distal end of cystic duct, three snares placed in all; the proximal and distal end of cystic duct and then cut in the middle of snares (Figure 1C). Confirmed no active haemorrhage and no biliary fistula. Consequently, cholecyst retracted through overtube applying forceps or basket (Figure 1D). Ultimately, autopsy was performed to check the dissection wound and adjacent viscera for injury.

## RESULTS

Transumbilical endoscopic cholecystectomy was successfully completed in the first and third pig, with minor bleedings (Tables 1, 2). The dissection times were 137 and 42 min, respectively. The total operation times were 167 and 69 min, respectively. The time required decreased with experience. And the lengths of resected specimen were 6.5 and 6.1 cm, respectively. Instillation of the fluid into the gallbladder bed produced edematous, distended tissue making separation safe and easy. Reliable ligation using double nylon loops insured the safety of cutting between the loops. There were no intraoperative complications or hemodynamic instability. At necropsy, the loops on the cystic duct and artery were secure and that neither bile nor blood leakage was observed from this site. Both the gallbladder bed and the liver bed were dry

(Figure 1E).

During the dissection in the second pig (Tables 1 and 2), unexpected gallbladder rupture occurred, and the perforation site was quickly clipped (Figure 1) after being identified. Subsequently, uncontrolled intraoperative bleeding occurred in the second case, leading to the operation failure.

## DISCUSSION

To date, most of pure NOTES procedures are still performed only in animals<sup>[2-4]</sup> due to several technical barriers. NOTES cholecystectomy has been successfully introduced into clinical application based on preliminary animal experimental researches. Several approaches has been reported in the literatures such as transgastric, transvaginal and transcolonic route, but most finished cholecystectomy with the assistance of laparoscopic instruments in humans<sup>[5-7]</sup>. Meanwhile, pure NOTES only has several case reports<sup>[8-10]</sup>. Transvaginal approach is the most widely used in the clinical practices and is considered<sup>[5]</sup> to be the best approach for NOTES surgery. However, it only apply to females with underlying complications like dyspareunia, infertility and pelvic adhesions<sup>[11,12]</sup>. Pugliese *et al*<sup>[5]</sup> showed no above-mentioned discomfort and incision related complications. Transgastric approach was originally studied<sup>[13,14]</sup>, but rarely applied to clinical practices because of technical difficulties<sup>[13,15,16]</sup>, to overcome these difficulties, the innovation of new technology and use of new devices has been reported in several literatures<sup>[9]</sup>. Furthermore, Marescaux *et al*<sup>[16]</sup> considered this approach for NOTES surgery would be

**Table 1** Transumbilical endoscopic cholecystectomy was successfully completed in the first and third pig, with minor bleedings

Animal	Sex	Weight (kg)	Time of pneumoperitoneum (min)	Time of dissection (min)	Time of ligation and resection (min)	Total time (min)
1	Female	30	2	137	28	167
2	Male	35	2	61 (incomplete)	-	-
3	Female	33	2	42	25	69

**Table 2** Experimental indicators in transumbilical endoscopic cholecystectomy

Animal	Blood loss (mL)	Perforation	Adjacent viscera injury	Size of specimen	Postoperative management
1	10	No	No	6.5 cm × 3.4 cm × 1.2 cm	Autopsy
2	50	Yes	Vascular injury	-	Autopsy
3	5	No	No	6.1 cm × 3.3 cm × 1.8 cm	Autopsy

the major route in future. Transcolonic NOTES similar to transvaginal approach has an advantage for upper abdominal organs, however, its major problem lies in potential contamination as a result of bacterial colonization. In this study, we performed cholecystectomy through umbilicus similar to single-port laparoscopic surgery. Truly speaking, to some extent it does not follow the true concept of NOTES. This approach compared to other approaches has no blind region in enterocoelia, in the other hand, it cannot support stable platform due to soft abdominal wall resulting in difficulties in manipulating endoscope<sup>[17]</sup>. Perforation occurred in our study owing to unstable platform. Therefore, experienced surgeon and endoscopist would be needed to cooperate with each other to complete cholecystectomy by using endoscope *via* umbilical approach. Any surgery require an appropriate and clear operative field vision achieved by manipulating overtube. We realized in practice that overtube was easy to displace because of its smoothness and flexibility which results in the lost operative field and had to re-reveal the gallbladder. So operator and assistant must keep tacit cooperation, the latter also plays a very important role who needs to regulate overtube from time to time in accordance with the procedure to support clear vision for operator which is significant in reducing operation time, avoiding accidental injury involving viscera, blood vessels and smoothly completing surgery. In a sentence, not a single perfect approach for the NOTES has been established till now. The development of endoscopic instruments and the evolution of technology have to be explored. Whether pure NOTES achieves full approval and advocacy from clinical practice depends on how the related challenges can be resolved practically.

The mechanism of water-jet is to employ high-pressure jet of water to cut materials fine, primarily used in manufacture. In 1982, water-jet was firstly introduced to medical application and performed liver resection<sup>[18,19]</sup>. German RAU firstly introduced professional designed water-jet into clinical application in 1990. From then on, its applications gradually extended to maxillofacial surgery, plastic surgery, urinary surgery, ophthalmosurgery, *etc*<sup>[20]</sup>. To date, its use is limited to the dissection of mesenchymal tissue and parenchymal organs<sup>[21,22]</sup>. In this field, German company ERBE is always ahead. Its

Helix-Hydro-jet device can perform precise, controllable tissue-selective (indicating water-rich tissue such as liver parenchyma) with minimal injury to the surrounding fibrous structures and has achieved favorable results in open and laparoscopic operations<sup>[18,23,24]</sup>. However, the above-mentioned Helix-Hydro-jet device cannot be passed through a standard working channel of the current flexible endoscope because its outer-diameter is larger than endoscopic operative channel, so do not match with NOTES procedures. ERBEJet2 water-jet system incorporates high-pressure water-jet with high-frequency electrocautery function with the characteristics of more flexibility, smaller size, easier handling, more precision and less foam formation compared with the precursor model Helix-Hydro-jet<sup>[25]</sup>. This new technology is mainly used to perform endoscopic submucosal dissection for gastrointestinal tumors. Studies showed that water-jet hybrid-knife could effectively shorten operation time and avoid endoscopic accessory-switch with lower haemorrhage and perforation rate, consequently improve the safety and efficiency of resection compared with conventional endoscopic knife<sup>[26-30]</sup>. Isayama *et al*<sup>[29]</sup> reported that transgastric pure NOTES successfully performed cholecystectomy using injection-dissection technology in the animal experiment with long operation time because the procedure of injection and dissection required the need of different instruments and keeps on frequently changing.

The work pressure of water-jet ranges from 30 to 80 bar. If lower than 30 bar, it cannot produce valid cushion, acting on mucous layer when more than 70 bar. While exceeding 100 bar may cause injury to deeper layer such as muscular layer<sup>[24,31]</sup>. Selective-tissue injection results in a selective deposition of mixed solution in the submucosa followed by mucosal elevation when the dissector placed directly on the mucous layer. High-pressure injection of solution into loose connective tissue between gallbladder and liver bed can produce a fluid lift cushion avoid the thermal damage to surrounding tissues, making dissection easier and safer<sup>[29]</sup>. In our study, neither uncontrolled hemorrhage of the liver fossa nor perforation of the gallbladder occurred, indicating the superiority of the ERBEJet2 water-jet system. What is worth mentioning, the lack of surgical triangulation, an important adjunct for

pure NOTES procedure, becomes less important, since the fluid lift cushion produced by the ERBEjet2 water-jet system facilitates dissection even there is no satisfactory traction or countertraction.

Refer to the selection of injecting Solution, perfect solution should have following advantages of being non-toxic to health, no injury to the injection site, low dispersion velocity, easy to access and cheap. Currently, normal saline solution is the most commonly employed with the drawback of being fast absorption, other selective solutions include hyaluronic acid, plasma expanders, gelatin, *etc.* Among them, hyaluronic acid is considered to be the best selection but most expensive<sup>[21,31,32]</sup>. Adding optimal adrenaline can delay solution absorption speed, prolong elevation time and decrease injection times. In our study, injection solution we used was saline solution that is easy to get and cheap. Furthermore, we had added methylene blue dye into saline solution in order to make the solution blue. Furthermore, it had helped clearing the boundaries between gallbladder's serosal layer and liver parenchyma by using high pressure injection with more precise and safe dissection along with maintaining prophylactic hemostasis. This can be seen from blood loss during the operation in our study.

In the functional cholecystectomy performed by Liu *et al.*<sup>[33]</sup>, the cystic duct was isolated and closed with an endoscopic clip. Since standard endoscopic clip may not be safe for use on the cystic duct as it has a hinge gap between the arms of the clip when deployed, in our study, the Olympus ligating device (Polyloop-detachable loop ligating device) was used to tie off the base of the gallbladder once exposed and isolated, and then the gallbladder was cut between the loops using the endoscopic polypectomy snare.

Calot's triangle complete exposure is very important for both the ligation of cystic duct, cystic artery and the avoidance of damage to biliary duct. Although double-channel endoscope has elevator, parallel correlation of double-channel cannot produce surgical triangulation resulting in limited orientation to manipulate. It is still difficult to ligate the cystic duct and cystic artery for pure NOTES that need more attention during the operation. The exposure of CVS (critical view of safety) can decrease the possibility of bile duct injury before ligating the cyst duct and artery<sup>[6]</sup>. We successfully performed pure NOTES cholecystectomy without laparoscopic support.

In conclusion, this study demonstrated the feasibility and safety of trans-umbilical NOTES cholecystectomy using the water-jet hybrid-knife. Due to absence of control study, the advantages of ERBEjet2 cannot be highlighted but possesses potential superiority. Therefore, further study will be designed to compare this new dissector with other endoscopic knife.

## COMMENTS

### Background

Laparoscopic cholecystectomy remains the gold standard for the treatment of benign gallbladder diseases, showing superiority such as better cosmetic

results, less postoperative pain, and shorter recovery time compared with open cholecystectomy. Under the guidance of the Holy Grail of minimal invasive surgery, namely, scarless surgery, natural orifice transluminal endoscopic surgery (NOTES) has made substantial progress. However, it is also technically difficult due to lack of appropriate devices and loss of triangulation.

### Research frontiers

To achieve a nonvisible scar, transumbilical endoscopic/laparoscopic cholecystectomy, single-incision laparoscopic surgery were proposed early or late.

### Innovations and breakthroughs

This is the first study evaluating feasibility and safety of a new method of cholecystectomy using NOTES transumbilical approach and water jet hybrid knife technology. The study demonstrated that the endoscopic water jet system can create a fine stream of unidirectional high-pressure saline solution to penetrate tissue, and instillation of the fluid into the gallbladder bed can produce edematous, distended tissue making separation safe and easy.

### Applications

Generally, transumbilical endoscopic cholecystectomy using a water-jet hybrid-knife is feasible and safe. How to avoid the injury of the cystic artery requires further investigation.

### Terminology

Hydraulic energy from pressurized fluid has been widely used in medical procedures. Instillation of fluid under high pressure into closed spaces can produce edematous, distended tissue, and the resulting increased weight and gravity force is enough to separate filmy avascular tissue without sharp dissection (electrosurgery).

### Peer review

This is a very interesting study about the feasibility and safety of Natural orifice trans-umbilical endoscopic cholecystectomy with a water-jet hybrid-knife in a non-survival porcine model. In this study, pure NOTES cholecystectomy was performed on three non-survival pigs, by transumbilical approach, with a double-channel flexible endoscope, and a water-jet hybrid-knife. The authors found that pure NOTES trans-umbilical cholecystectomy with a water-jet hybrid-knife appear to be feasible and safe.

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## Estrogen improves the hyperdynamic circulation and hyporeactivity of mesenteric arteries by alleviating oxidative stress in partial portal vein ligated rats

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### Abstract

**AIM:** To evaluate the effects of estrogen (E2) on systemic and splanchnic hyperdynamic circulation in portal hypertensive rats.

**METHODS:** Fifty castrated female Sprague-Dawley rats were divided into five groups: sham operation (SO), partial portal vein ligation (PPVL) + placebo (PLAC), PPVL + E2, PPVL + ICI and PPVL + E2 + ICI. Hemodynamic measurements were performed using ultrasonography. Mesenteric arteriole contractility in response to norepinephrine was determined using a vessel perfusion system. Oxidative stress in the mesenteric artery was investigated by *in situ* detection of the superoxide anion ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ) concentrations.

**RESULTS:** Treatment with E2 resulted in a significant decrease of portal pressure ( $P < 0.01$ ) and portal venous inflow ( $P < 0.05$ ), and higher systemic vascular resistance ( $P < 0.05$ ) and splanchnic arteriolar resis-

tance ( $P < 0.01$ ) in PPVL + E2 rats compared to PPVL + PLAC rats. In the mesenteric arterioles of PPVL + E2 rats, the dose-response curve was shifted left, and the  $EC_{50}$  was decreased ( $P < 0.01$ ). E2 reduced  $O_2^{\cdot-}$  production and  $H_2O_2$  concentration in the mesenteric artery. However, ICI182, 780 reversed the beneficial effects of E2, therefore, the systemic and splanchnic hyperdynamic circulation were more deteriorated in ICI182, 780-treated rats.

**CONCLUSION:** Treatment with estrogen improved the systemic and splanchnic hyperdynamic circulation in PPVL rats, in part due to the alleviation of oxidative stress.

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**Key words:** Estrogen; Hyperdynamic circulation; Partial portal vein ligation; Oxidative stress

**Core tip:** Vascular hyporeactivity is affected by gender and estrogen. The aim of the present study was to investigate whether estrogen could attenuate the severity of hyperdynamic circulation and the underlying mechanism in pre-hepatic portal hypertensive rats without cirrhosis, with a focus on oxidative stress. The authors proposed that treatment with estrogen could improve the systemic and splanchnic hyperdynamic circulation in partial portal vein ligation rats, in part due to the alleviation of oxidative stress.

Zhang B, Zhang CG, Zhou QB, Chen W, Wu ZY. Estrogen improves the hyperdynamic circulation and hyporeactivity of mesenteric arteries by alleviating oxidative stress in partial portal vein ligated rats. *World J Gastroenterol* 2013; 19(40): 6863-6868 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6863.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6863>

## INTRODUCTION

Portal hypertension (PHT) is a major complication of liver cirrhosis and is associated with the development of hyperdynamic circulation, characterized by elevated portal pressure (PP), increased splanchnic (small intestine) blood flow, increased cardiac output, and development of an extensive network of portosystemic collaterals<sup>[1]</sup>. PP is determined by extrahepatic factors such as portal blood flow and collateral resistance in addition to intrahepatic resistance. Therefore, it would be triggered by persistent splanchnic vasodilation. Functional changes can be found in the portal hypertensive splanchnic vasculature, including splanchnic vasodilation and decreased responsiveness to vasoconstrictors as a result of endothelial dysfunction and impaired activation of vasoconstrictive mechanisms<sup>[2]</sup>. Although the levels of vasoconstrictors such as norepinephrine (NE) and angiotensin- II (Ang- II) are increased in the blood circulation and are accompanied by enhanced sympathetic excitability in PHT, the splanchnic vasculature remains dilated<sup>[3]</sup>. Hyporeactivity of the artery in response to vasoconstrictors plays a key role in blood vessel dilation and hyperdynamic circulation<sup>[4,5]</sup>. Vascular hyporeactivity has been observed in PHT animals in both the systemic circulation and in the mesenteric artery, and it is also affected by gender and estrogen<sup>[6]</sup>. In addition, experimental studies have shown that estradiol (E2) reduces PP and increases hepatic blood flow in castrated and cirrhotic model rats, but the effect is significantly inhibited by the estrogen antagonist ICI182, 780<sup>[7,8]</sup>.

Both acute and chronic liver diseases are characterized by an increased formation of reactive oxygen species (ROS), as indicated by increased superoxide anion in the whole blood of cirrhotic patients with decompensated liver and increased urinary F2-isoprostanes and lipoperoxidation and decreased superoxide dismutase in experimental PHT models<sup>[9]</sup>. Increased oxidative stress may lead to impaired endothelium dysfunction and be involved in the pathogenesis of PHT. It has therefore been a subject of interest<sup>[10,11]</sup>.

The aim of the present study was to investigate whether estrogen could attenuate the severity of hyperdynamic circulation and the underlying mechanism in PHT rats without cirrhosis, with a focus on oxidative stress. Thus, to exclude the hepatoprotective and antifibrogenic effects of estrogen, the PHT was induced by partial portal vein ligation (PPVL) but not by the injection of hepatotoxic drugs, such as CCl<sub>4</sub> and dimethylnitrosamine, or by bile duct ligation.

## MATERIALS AND METHODS

### *Animal model and treatment*

This animal study was approved by the local animal ethics committee of Renji Hospital and performed according to the guidelines of the Laboratory Animal Care and Use Committee at School of Medicine, Shanghai Jiao Tong University (Shanghai, China). Fifty female Sprague-Dawley rats weighing 200-220 g underwent ovariectomy

before the study. Three weeks after the initial surgery, the rats underwent PPVL or sham operation (SO) as previously described by Reiberger *et al*<sup>[1]</sup> and were divided into five groups: SO, PPVL + placebo (PLAC), PPVL + E2, PPVL + ICI and PPVL + E2 + ICI. Briefly, under ketamine anesthesia (intramuscular injection of 10 mg/100 g body weight), the portal vein was isolated, and stenosis was induced by a single ligature of 3-0 silk placed around both the portal vein and a 20-gauge blunt-tipped needle. The needle was then removed, leaving a calibrated stenosis of the portal vein. Portal hypertension was considered present at 14 d after surgery. In SO animals, the portal vein was isolated and similarly manipulated but not ligated. Starting on the day of surgery, the PPVL + E2, PPVL + ICI and PPVL + E2 + ICI groups were subcutaneously administered  $\beta$ -estradiol (2  $\mu$ g/100 g body weight/d, R and D Systems, United States) or/and ICI182, 780 (2  $\mu$ g/100 g body weight/d, R and D Systems, United States) for 14 d. The SO and PPVL groups were subcutaneously administered the same volume of the placebo (ethanol).

### *Hemodynamic measurements*

Under anesthesia induced by an intramuscular injection of ketamine (10 mg/100 g body weight), the velocity and inside diameter of the portal vein (PV) proximal to the ligation and stroke volume (SV) were obtained using a Vevo770 High-Resolution Imaging System (Visual Sonics, Canada). A 22 G catheter filled with heparin saline was inserted into the femoral artery to obtain the mean arterial pressure (MAP) and heart rate (HR). Another 22 G catheter was introduced into the portal vein to measure PP, and one was inserted into the inferior vena cava to measure central venous pressure (CVP) after making an incision at the midline of the abdomen. All parameters were recorded using an SP840 pressure transducer and a multichannel recorder (Philips, United States).

Cardiac output (CO), cardiac index (CI), PV blood flow, portal venous inflow (PVI), systemic vascular resistance (SVR) and splanchnic arteriolar resistance (SAR) were calculated as follows: CO = SV  $\times$  HR; CI = CO  $\times$  100/body weight (g); PVI = PV blood flow  $\times$  100/body weight (g); SVR = (MAP - CVP)/CI; SAR = (MAP - PP)/PVI. PV blood flow and CO were normalized by body weight and presented as CI and PVI. Resistances in the vascular systems were calculated from the ratio between the perfusion pressure (*P*) and blood flow (*Q*) of each vascular territory.

### *Determination of mesenteric arteriole reactivity to NE*

Following the determination of hemodynamic measurements, the mesenteric arteries and the mesentery were removed. Using an SMZ-168 dissecting microscope (Motic, China), the third-order arterioles in the mesentery were carefully dissected and transferred to a vascular perfusion system containing a 3-(*N*-morpholino) propanesulfonic acid-buffered physiological salt solution (MOPS-PSS, 0-4 °C, pH 7.4; NaCl 145 mmol/L, KCl 5.0 mmol/L, CaCl<sub>2</sub> 2.0 mmol/L, MgSO<sub>4</sub> 1.0 mmol/L, NaH<sub>2</sub>PO<sub>4</sub> 1.0

**Table 1 Hemodynamic data of the five animal groups and maximum contraction and EC50 in the five groups**

	SO	PPVL + PLAC	PPVL + E2	PPVL + ICI	PPVL + E2 + ICI
Body weight (g)	248 ± 15	261 ± 22	245 ± 18	231 ± 23 <sup>a,c</sup>	244 ± 13
HR (bpm)	440 ± 32	412 ± 25 <sup>a</sup>	415 ± 35 <sup>a</sup>	419 ± 21 <sup>a</sup>	406 ± 28 <sup>a</sup>
MAP (mmHg)	95 ± 6	85 ± 11 <sup>a</sup>	88 ± 9 <sup>a</sup>	89 ± 5 <sup>a</sup>	84 ± 8 <sup>a</sup>
PP (mmHg)	4.6 ± 1.2	14.9 ± 0.9 <sup>b</sup>	12.2 ± 1.6 <sup>b</sup>	14.2 ± 1.5 <sup>b</sup>	13.9 ± 1.3 <sup>b</sup>
PV flow (mL/min)	8.52 ± 2.3	12.71 ± 3.1	9.76 ± 2.8	11.87 ± 3.5	13.04 ± 3.8
PVI (mL/min/100 g)	3.43 ± 1.2	4.87 ± 1.6 <sup>a</sup>	3.98 ± 1.4 <sup>c</sup>	5.14 ± 1.8 <sup>a</sup>	5.34 ± 1.9 <sup>a</sup>
CO (mL · min <sup>-1</sup> )	54.68 ± 4.9	61.79 ± 5.9	52.07 ± 5.1	56.82 ± 5.6	57.91 ± 6.0
CI (mL/min/100 g)	22.05 ± 1.9	23.67 ± 2.1	21.25 ± 2.0	24.60 ± 2.2	23.79 ± 2.5
SVR (mmHg·min-100 g/mL)	4.08 ± 0.48	3.38 ± 0.35 <sup>b</sup>	3.95 ± 0.41 <sup>c</sup>	3.50 ± 0.38 <sup>a</sup>	3.32 ± 0.33 <sup>a</sup>
SAR (mmHg·min-100 g/mL)	26.33 ± 2.7	14.39 ± 2.2 <sup>b</sup>	19.29 ± 1.8 <sup>b,d</sup>	14.94 ± 1.7 <sup>b</sup>	13.12 ± 1.5 <sup>b</sup>
Emax	75.18% ± 4.52%	50.47% ± 3.48% <sup>a</sup>	70.65% ± 2.42% <sup>a,d</sup>	51.37% ± 4.12% <sup>b</sup>	54.33% ± 4.71% <sup>b</sup>
EC50 (10 <sup>-6</sup> mol/L)	2.77 ± 0.74	5.27 ± 0.88 <sup>b</sup>	3.77 ± 0.69 <sup>b,d</sup>	4.89 ± 0.76 <sup>b</sup>	3.85 ± 0.52 <sup>b,d</sup>

Significant differences are marked with superscripts showing the *P* values. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs sham operation (SO); <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 vs partial portal vein ligation (PPVL) + placebo (PLAC); E2: Estrogen; HR: Heart rate; MAP: Mean arterial pressure; PP: Portal pressure; PV: Portal vein; CO: Cardiac output; CI: Cardiac index; PVI: Portal venous inflow; SVR: Systemic vascular resistance; SAR: Splanchnic arteriolar resistance.

mmol/L, glucose 5.0 mmol/L, pyruvate 2.0 mmol/L, EDTA 0.02 mmol/L and MOPS 3.0 mmol/L). A glass micropipette containing MOPS-PSS (top diameter, 50 μm) was inserted into one end of the arteriole and fixed with 11-0 single strands. Blood was flushed out at a perfusion pressure of 8 mmHg. Another glass micropipette was then inserted into the other end of the arteriole and fixed. The two glass micropipettes were suspended in organ baths containing 60 mL of MOPS-PSS (37 °C, pH 7.4). The arteriole was equilibrated under a pressure of 80 mmHg for 30 min prior to the experiments. After the equilibration period, cumulative NE concentration response curves (10<sup>-8</sup> mol/L-10<sup>-4</sup> mol/L) were obtained by increasing the concentration in quarter-log increments. The inner diameter was measured using a BA310 microscope camera system (Motic, China). The vasoconstriction rate and the logarithm of the NE concentration were used as the vertical axis and the abscissa, respectively.

### Superoxide anion detection

The oxidative fluorescent dihydroethidium (DHE, Sigma, United States) was used to evaluate in situ production of superoxide anion (O<sub>2</sub><sup>•-</sup>)<sup>[12]</sup>. Frozen, enzymatically intact, 10-μm-thick sections of the superior mesenteric arteries were incubated with DHE (10 μmol/L) in PBS for 30 min at 37 °C in a humidified chamber protected from light. DHE is freely permeable to cells and is oxidized in a reaction with O<sub>2</sub><sup>•-</sup> to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. Images were obtained with an IX71 fluorescence microscope (Olympus, Japan).

### Hydrogen peroxide determination

The level of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was measured using a hydrogen peroxide assay kit (Abcam, United States). In the presence of horseradish peroxidase (HRP), the OxiRed Probe reacts with H<sub>2</sub>O<sub>2</sub> to produce product with color. The superior mesenteric artery was cleaned of connective tissue, precipitated with RIPA (Beyotime, China; 200 μL RIPA/20 mg tissue) for 15 min and then centrifuged for 15 min at 1000 g. A total of 5 μL of

the supernatant was diluted with 46 μL of assay buffer, mixed with 50 μL of the Reaction Mix (assay buffer 46 μL, OxiRed Probe 2 μL, HRP 2 μL) and then incubated at room temperature for 10 min. The OD570 nm was read with a Synergy 4 Multi-Mode Microplate Reader (BioTek, United States), and the H<sub>2</sub>O<sub>2</sub> concentration was calculated according to a standard concentration curve.

### Statistical analysis

The change in the reactivity of the mesenteric arteriole in response to NE was presented as a dose-response curve, which was fitted by a nonlinear regression analysis (GraphPad Software Inc., San Diego, CA, United States). Maximal responses (Emax) and effective concentrations causing half maximum responses (EC<sub>50</sub>, calculated by regression analysis) were obtained from concentration response curves. Values are expressed as the means ± SD. Statistical comparisons were performed using one-way ANOVA. *P* values < 0.05 were considered significant. All statistical analyses were performed using the Statistical Package for the Social Sciences 13.0 (SPSS Inc., United States).

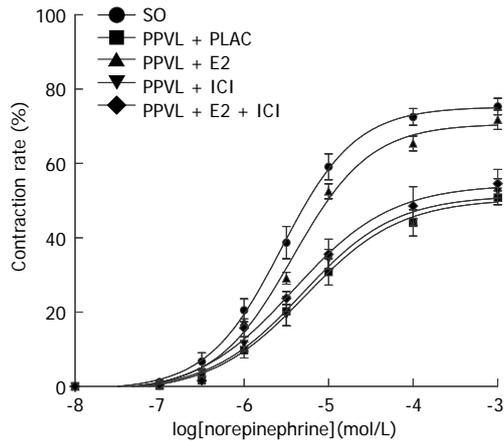
## RESULTS

### Hemodynamics

Compared to SO, PPVL resulted in a lowered HR (*P* < 0.05) and MBP (*P* < 0.05) and significantly increased PP (*P* < 0.01) in the four groups of prehepatic PHT. However, measurement of MAP and HR revealed no significant differences between the four PPVL groups (*P* > 0.05). PP significantly decreased by 18.5% in PPVL + E2 rats with a mean PP of 12.2 ± 1.6 mmHg compared to a mean PP of 14.9 ± 1.4 mmHg in PPVL + PLAC rats (*P* < 0.01) (Table 1).

PVI was significantly increased in the four groups of rats that underwent PPVL compared to the corresponding SO group (*P* < 0.01). Treatment with E2 resulted in a reduction in PVI in PPVL + E2 rats compared to PPVL + PLAC rats (3.98 ± 1.4 mL/min per 100 g vs 4.87 ± 1.6 mL/min per 100 g, *P* < 0.05) (Table 1).

Although CI was slightly increased in the four groups



**Figure 1** Dose-response curves for isolated rat mesenteric arteriole contractility in response to norepinephrine at different concentrations. The dose-response curves in the four partial portal vein ligation (PPVL) groups were lowered and shifted to the right compared to the sham operation (SO) group. Treatment with estrogen (E2) reversed the curve to left, whereas ICI182, 780 decreased the response to norepinephrine of arterioles treated with E2. PLAC: Placebo.

of PPVL rats compared to the SO group, there were no significant differences among the five different groups ( $P > 0.05$ ). SVR was lower in the four groups of PPVL rats compared to SO rats due to the decreased MBP ( $P < 0.05$ ), which was increased 16.9% by E2 treatment in PPVL + E2 rats compared to PPVL + PLAC rats ( $3.95 \pm 0.41$  mmHg·min·100 g/mL *vs*  $3.38 \pm 0.35$  mmHg·min·100 g/mL,  $P < 0.05$ ) (Table 1).

SAR was significantly lower in the four groups of PPVL rats compared to SO rats ( $P < 0.01$ ). Rats treated with E2 showed significantly higher SAR, with a 34.1% increase compared to PPVL + PLAC rats ( $19.29 \pm 1.8$  mmHg·min·100 g/mL *vs*  $14.39 \pm 2.2$  mmHg·min·100 g/mL,  $P < 0.01$ ) (Table 1).

ICI182, 780 treatment alone did not influence PP, PVI, CI, SVR or SAR compared to PPVL + PLAC rats, except that the body weight was slightly lower ( $P < 0.05$ ). However, ICI182, 780 reversed the beneficial effects of E2 on PVI, CI, SVR and SAR. Thereby, the systemic circulation and splanchnic hyperdynamic circulation were more deteriorated (Table 1).

### Contractility of isolated rat mesenteric arterioles in response to NE

Compared with the SO group, the dose-response curve of the mesenteric arteriole in response to NE was lower and shifted right, with decreased  $E_{max}$  ( $P < 0.01$ ) and increased  $EC_{50}$  ( $P < 0.01$ , Figure 1; Table 1) in the four PPVL groups. In the mesenteric arterioles of PPVL rats treated with E2, the dose-response curve was shifted left, and the  $EC_{50}$  was decreased ( $P < 0.01$ ), compared with the PPVL + PLAC rats. ICI182, 780 alone did not influence the dose-response curve or  $EC_{50}$  compared to PPVL rats treated with placebo ( $P > 0.05$ ). However, ICI182, 780 decreased the response to NE of arterioles treated with E2, thereby the dose-response curve was shifted right.

### Superoxide anion production

Low-intensity fluorescence was observed throughout SO vessels, confirming that all layers of the normal vessel produced low amounts of  $O_2^{\bullet-}$ . Fourteen d after the surgery,  $O_2^{\bullet-}$  production was increased in the four groups of PPVL rats (Figure 2). However, the staining was particularly weak in PPVL + E2 rats, and the administration of ICI182, 780 blocked the beneficial effect that E2 provided.

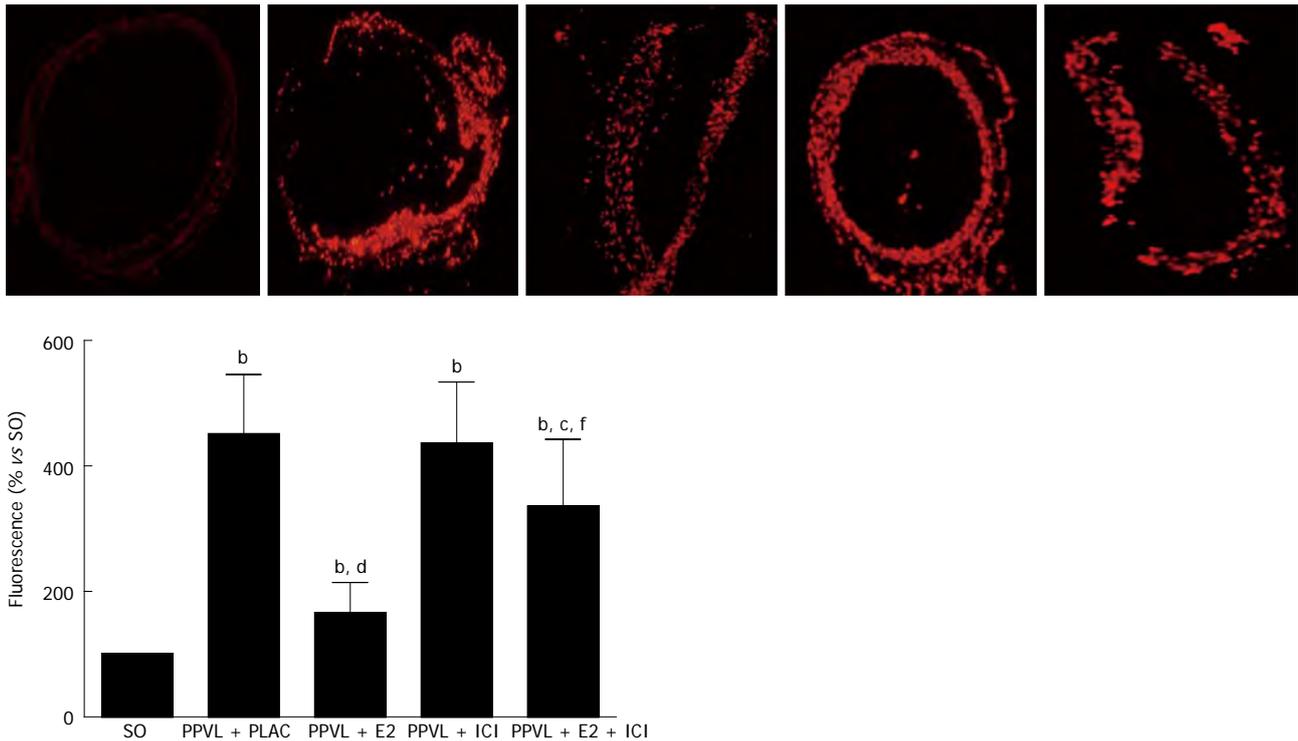
### Hydrogen peroxide production

In the superior mesenteric arteries,  $H_2O_2$  generation was nearly 2.5-fold higher in PPVL + PLAC rats than in SO rats ( $8.2 \pm 1.5$   $\mu$ mol/L *vs*  $3.3 \pm 0.9$   $\mu$ mol/L,  $P < 0.01$ ; Figure 3). Exogenous E2 reduced  $H_2O_2$  levels in PPVL + E2 rats when compared with those in PPVL + PLAC rats ( $4.9 \pm 1.0$   $\mu$ mol/L *vs*  $8.2 \pm 1.5$   $\mu$ mol/L,  $P < 0.01$ ). The  $H_2O_2$  levels in mesenteric arteries treated with ICI182, 780 alone were similar to those found in PPVL + PLAC rats ( $7.9 \pm 1.8$   $\mu$ mol/L *vs*  $3.3 \pm 0.9$   $\mu$ mol/L,  $P < 0.01$ ). However, the administration of ICI182, 780 to PPVL + E2 rats suppressed the antioxidant effect of E2 ( $6.6 \pm 1.3$   $\mu$ mol/L *vs*  $4.9 \pm 1.0$   $\mu$ mol/L,  $P < 0.05$ ).

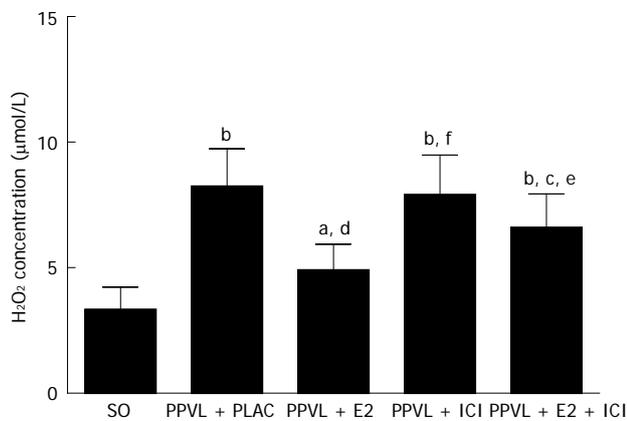
## DISCUSSION

Recent studies have already provided evidence of the importance of vascular hyporeactivity in the development and maintenance of PHT. Aortic ring hyporeactivity has been investigated both in bile duct ligation and  $CCl_4$  induced cirrhotic models<sup>[13-15]</sup>. Ferlitsch *et al*<sup>[16]</sup> have shown that forearm artery responses to NE and Ang-II are decreased in patients with cirrhosis. Clear sex differences have also been observed in the vasoconstrictor responsiveness of aortic rings from rats with and without PHT<sup>[17]</sup>. In contrast to male rats, PHT does not induce vascular hyporesponsiveness in female rats. Estrogen has been shown to be effective in animal models of established PHT with cirrhosis by suppressing hepatic fibrosis and relaxation of the hepatic sinusoid<sup>[7,18]</sup>. However, the efficacy of estrogen in the setting of *de novo* PHT and the underlying mechanism had not been characterized in detail. To exclude the effect of cirrhosis on oxidative status and inflammatory cytokines, we established this animal model of prehepatic PHT to further describe both the hemodynamic and anti-oxidant effects of estrogen treatment in the PPVL rat model. The hypothesis of our study was that estrogen could reverse the severity of hyperdynamic circulation and the vascular hyporeactivity of the mesenteric arteries by alleviating oxidative stress in portal hypertensive rats without cirrhosis in which liver function was normal.

The results of our study suggested that mesenteric arteriole sensitivity and contractility in response to NE were decreased in PPVL rats, indicating the hyporesponsiveness of the splanchnic vessels to vasoconstrictors, in accordance with deteriorative splanchnic hemodynamics. Treatment with E2 significantly ameliorated the hyperdynamic splanchnic circulation in PPVL rats. The reduction



**Figure 2** *In situ* detection of superoxide anion. Fluorescence micrographs of superior mesenteric arteries stained with the  $O_2^{\cdot-}$ -sensitive dye DHE (red fluorescence) were obtained from sham operation (SO) and partial portal vein ligation (PPVL) rats at 14 d after surgery. Images were acquired at identical settings. <sup>b</sup> $P < 0.01$  vs SO; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs PPVL + placebo (PLAC); <sup>f</sup> $P < 0.01$  vs PPVL + estrogen (E2).



**Figure 3** Hydrogen peroxide determination. The hydrogen peroxide concentrations of the superior mesenteric arteries were obtained from sham operation (SO) and partial portal vein ligation (PPVL) rats 14 d after surgery. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs SO; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs PPVL + placebo (PLAC); <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$  vs PPVL + estrogen (E2).

in splanchnic blood inflow represented by decreased PVI could be explained by an improved contractile reaction of the splanchnic vessels to the vasoconstrictor and a subsequently increased SAR. Additionally, the production of ROS was decreased in PPVL-E2 rats compared to PPVL-SA rats, indicating a profound amelioration of oxidative stress and corresponding to the improvement of systemic and splanchnic hyperdynamic circulation.

The fact that PPVL resulted in oxidant injury was first demonstrated by Fernando *et al.*<sup>[18]</sup>, who concluded that the formation of ROS may be important in the patho-

genesis of hemodynamic changes and that anti-oxidants can ameliorate oxidant injury and prevent the development of hyperdynamic circulation<sup>[19]</sup>. Estrogen played a protective role by acting as an antioxidant, which granted it action as a scavenger of free radicals, decreasing the formation of ROS. In castrated rats, significant increases in the activity of antioxidant enzymes were observed, which may have occurred to compensate for the absence of circulating E2 promoted by castration; even then, it was not effective in reducing lipid peroxidase levels<sup>[8,20,21]</sup>. Estrogen replacement can reverse this effect, reducing lipid peroxidase to the values of control animals<sup>[21]</sup>. In addition, estrogen stimulates eNOS expression in SECs and increases NO production, contributing to a reduction in portal pressure in a model of intrahepatic PHT<sup>[7]</sup>.

In summary, we conclude that improvements in oxidative stress after estrogen administration manifest as a functional improvement in the contractile response to vasoconstrictors. Indeed, we observed improvements in both the systemic and splanchnic hyperdynamic circulation. Further studies on the clinical administration of estrogen should be performed.

## COMMENTS

### Background

Portal hypertension, a major complication of liver cirrhosis, is associated with the development of hyperdynamic circulation and would be triggered by persistent splanchnic vasodilation. Functional changes can be found in the portal hypertensive splanchnic vasculature, including splanchnic vasodilation and decreased responsiveness to vasoconstrictors as a result of endothelial dysfunction and impaired activation of vasoconstrictive mechanisms.

**Research frontiers**

Increased oxidative stress may lead to impaired endothelium dysfunction and be involved in the pathogenesis of portal hypertension. It has therefore been a subject of interest

**Innovations and breakthroughs**

The authors found that improvements in oxidative stress after estrogen administration manifest as a functional improvement in the contractile response of mesenteric arteries to vasoconstrictors in partial portal vein ligation (PPVL) rats.

**Applications**

The authors proposed that treatment with estrogen could improve the systemic and splanchnic hyperdynamic circulation in PPVL rats.

**Peer review**

The manuscript is an interesting manuscript with novel observations. The authors delineated the effect of estrogen treatment on the systemic and splanchnic hyperdynamic circulation in portal hypertensive rats. The findings are straight forward and manuscript is well written and nicely discussed.

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**L- Editor** A **E- Editor** Zhang DN



## Transarterial embolization for massive gastrointestinal hemorrhage following abdominal surgery

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Author contributions: Zhou CG and Shi HB designed the study and wrote the manuscript; Liu S and Yang ZQ performed the transarterial embolization procedures; Zhou CG, Zhao LB, Xia JG and Zhou WZ assisted in patient recruitment and interviews; Shi HB and Li LS corrected the manuscript.

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### Abstract

**AIM:** To evaluate the clinical results of angiography and embolization for massive gastrointestinal hemorrhage after abdominal surgery.

**METHODS:** This retrospective study included 26 patients with postoperative hemorrhage after abdominal surgery. All patients underwent emergency transarterial angiography, and 21 patients underwent emergency embolization. We retrospectively analyzed the angiographic features and the clinical outcomes of transcatheter arterial embolization.

**RESULTS:** Angiography showed that a discrete bleeding focus was detected in 21 (81%) of 26 patients. Positive angiographic findings included extravasations of contrast medium ( $n = 9$ ), pseudoaneurysms ( $n = 9$ ), and fusiform aneurysms ( $n = 3$ ). Transarterial embolization was technically successful in 21 (95%) of 22 patients. Clinical success was achieved in 18 (82%) of

22 patients. No postembolization complications were observed. Three patients died of rebleeding.

**CONCLUSION:** The positive rate of angiographic findings in 26 patients with postoperative gastrointestinal hemorrhage was 81%. Transcatheter arterial embolization seems to be an effective and safe method in the management of postoperative gastrointestinal hemorrhage.

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**Key words:** Transcatheter arterial embolization; Postoperative hemorrhage; Complications; Surgery

**Core tip:** Postoperative gastrointestinal hemorrhage is a potentially fatal complication after abdominal surgery. It is difficult for surgeons to deal with it. Reoperation is often difficult or even unsuccessful in patients with postoperative hemorrhage, especially those with two or more previous abdominal operations, due to the anatomical inaccessibility of the arteries, postoperative adhesions, and inflammatory reactions. This study showed that transcatheter embolization was a useful microinvasive treatment option for the identification and occlusion of a massive bleeding site after abdominal surgery.

Zhou CG, Shi HB, Liu S, Yang ZQ, Zhao LB, Xia JG, Zhou WZ, Li LS. Transarterial embolization for massive gastrointestinal hemorrhage following abdominal surgery. *World J Gastroenterol* 2013; 19(40): 6869-6875 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6869.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6869>

### INTRODUCTION

Postoperative gastrointestinal hemorrhage is a potentially

fatal complication after abdominal surgery. It prolongs hospital stay, requires urgent radiological or surgical intervention, and increases mortality after abdominal surgery. The incidence of postoperative gastrointestinal hemorrhage after abdominal surgery is low, but increases with an increase in surgical procedures, severity of illness, and comorbid conditions. The incidence of postoperative gastrointestinal hemorrhage has been reported as 0.4%-4% in a recent series<sup>[1-7]</sup>, and 2%-18% in earlier series<sup>[8-14]</sup>. Although recent studies have shown that its mortality has decreased, it remains a serious and life-threatening condition.

Traditionally, open ligation or excision has been considered to be the first-line therapeutic option for patients with massive gastrointestinal hemorrhage after abdominal surgery. However, the bleeding site is difficult to establish because of local inflammatory response after abdominal surgery. In addition, patients with postoperative gastrointestinal hemorrhage are reported to be poor candidates for emergency surgery because of cicatrization and friability of postoperative tissues<sup>[15]</sup>.

Transcatheter angiographic embolization is a less invasive procedure that is known to be a safe and effective treatment to control massive gastrointestinal hemorrhage. With the development of endovascular techniques over the past decade, transarterial embolization has been widely used clinically for treatment of postoperative gastrointestinal hemorrhage after abdominal surgery, despite the possibility of gastrointestinal infarction<sup>[16-19]</sup>.

In this study, we retrospectively reviewed and analyzed the angiographic findings and clinical outcomes of transarterial angiography and embolization in 26 patients with postoperative hemorrhage after abdominal surgery.

## MATERIALS AND METHODS

This study was approved by our institutional review board, and all patients gave their informed consent before the procedure. This study included 26 patients (22 male and 4 female) who underwent emergency transarterial angiography and embolization for postoperative hemorrhage after abdominal surgery between August 2007 and April 2012 at our hospital. The mean age was 57.2 years (range, 35-86 years). The average time of onset of postoperative hemorrhage was 27.7 d (range, 3-65 d). The abdominal surgery included surgery for gastric carcinoma ( $n = 13$ ), pancreatic head carcinoma ( $n = 2$ ), common bile duct carcinoma ( $n = 2$ ), duodenal papilla carcinoma ( $n = 2$ ), ascending colon carcinoma ( $n = 1$ ), severe pancreatitis ( $n = 1$ ), gallbladder carcinoma ( $n = 1$ ), cholangiolithiasis ( $n = 1$ ), and intra-abdominal abscess ( $n = 1$ ), as well as splenomegaly ( $n = 1$ ), and mesenteric torsion ( $n = 1$ ). Clinical presentations included hematemesis, hemochezia/melena, and bleeding from surgical drains. The volume of bleeding was  $> 1$  L in 24 h.

The diagnostic angiography was performed via transfemoral approach, using a 5-F angiographic catheter (Cook, Bloomington, IN, United States) and a 5-F sheath

(Terumo, Tokyo, Japan). In all cases, celiac and superior mesenteric angiography was routinely performed to detect the bleeding points. If this did not detect any bleeding points, inferior mesenteric angiography was performed. Hemorrhage was diagnosed based on the presence of extravasation of contrast agent, a pseudoaneurysm, and a fusiform aneurysm on angiography. Immediately after bleeding points were identified, transarterial embolization was performed with microcoils (Cook) and/or gelatin sponge (gelfoam particles) through a coaxial 2.7-F microcatheter (Terumo). Transarterial embolization was also performed with gelfoam in one patient without positive angiographic findings. Technical success was defined as devascularization of the target vessels on postembolization angiography. Clinical success was defined as cessation of clinical symptoms (including melena, hematemesis, and hemochezia), and no requirement for subsequent hemostatic interventions (such as surgery, endoscopic therapy, and second embolization).

The diameter of microcoils we used was from 2 to 6 mm. The length of microcoils was from 3 to 8 cm. The diameter of gelfoam particles was approximately 1 mm. Clinical follow-up period was 3 mo in all patients.

## RESULTS

Fifteen patients presented with hematemesis/melena, and 11 patients presented with bleeding from surgical drains. Twenty-two patients had signs of shock (systolic blood pressure  $< 100$  mmHg and pulse rate  $> 100$  beats/min). The clinical features and angiographic findings are summarized in Table 1. Results of transarterial embolization are summarized in Table 2.

Bleeding points near the surgical fields were detected by angiography in 21 (81%) of 26 patients. Positive angiographic findings included extravasation of contrast medium ( $n = 9$ ) (Figure 1), pseudoaneurysms ( $n = 9$ ) (Figures 2 and 3), and fusiform aneurysms ( $n = 3$ ). Extravasation of contrast medium was observed from the jejunal artery ( $n = 2$ ), gastroduodenal artery ( $n = 2$ ), right hepatic artery and gastroduodenal artery ( $n = 1$ ), great pancreatic artery ( $n = 1$ ), inferior pancreaticoduodenal artery ( $n = 1$ ), dorsal pancreatic artery ( $n = 1$ ), and ileocolic artery ( $n = 1$ ). Pseudoaneurysms were found in the gastroduodenal artery ( $n = 3$ ), common hepatic artery ( $n = 1$ ), right hepatic artery ( $n = 1$ ), inferior pancreaticoduodenal artery ( $n = 1$ ), splenic artery and right gastroepiploic artery ( $n = 1$ ), jejunal artery ( $n = 1$ ), and superior rectal artery ( $n = 1$ ). The fusiform aneurysms were identified in the gastroduodenal artery ( $n = 2$ ), and the proper hepatic artery ( $n = 1$ ).

Transarterial embolization was performed in 20 of 21 patients with positive angiographic findings and one patient without positive angiographic findings. The embolized arteries are summarized in Table 2. Transarterial embolization of bleeding arteries was performed using a combination of microcoils and gelatin sponge in six cases, microcoils in 13 cases, and gelatin sponge in two cases. Transarterial embolization was not performed in

**Table 1** Clinical features of patients with postoperative hemorrhage after abdominal surgery

Case	Sex	Age (yr)	Diseases	Surgical procedure	Interval from operation to bleeding (d)	Clinical presentations
1	Male	61	Gastric carcinoma	Gastrectomy	11	Haematemesis/melena
2	Male	72	Pancreatitis	Pancreatitis necrosectomy	15	Bleeding from drain
3	Male	46	Duodenal papilla carcinoma	Pancreaticoduodenectomy	38	Bleeding from drain
4	Male	64	Gastric carcinoma	Gastrectomy	14	Bleeding from drain
5	Male	37	Gastric carcinoma	Gastrectomy	27	Haematemesis/melena
6	Female	44	Gastric carcinoma	Gastrectomy	18	Bleeding from drain
7	Male	51	Gastric carcinoma	Gastrectomy	20	Haematemesis/melena
8	Male	35	Gastric carcinoma	Gastrectomy	28	Haematemesis/melena
9	Male	41	Gastric carcinoma	Gastrectomy	64	Haematemesis/melena
10	Male	56	Pancreatic carcinoma	Pancreaticoduodenectomy	10	Haematemesis/melena
11	Female	45	Gallbladder carcinoma	Extended cholecystectomy	25	Haematemesis/melena
12	Male	86	Ascending colon carcinoma	Right hemicolectomy	34	Hematochezia/melena
13	Male	59	Gastric carcinoma	Gastrectomy	50	Haematemesis/melena
14	Male	69	Gastric carcinoma	Gastrectomy	49	Bleeding from drain
15	Male	61	Intra-abdominal abscess	Excision of Intra-abdominal abscess	3	Bleeding from drain
16	Male	65	Common Bile duct carcinoma	Pancreaticoduodenectomy	29	Bleeding from drain
17	Male	73	Gastric carcinoma	Gastrectomy	38	Hematochezia/melena
18	Male	60	Bile duct carcinoma	Pancreaticoduodenectomy	29	Bleeding from drain
19	Female	62	Gastric carcinoma	Gastrectomy	21	Haematemesis/melena
20	Male	80	Duodenal papilla carcinoma	Pancreaticoduodenectomy	8	Bleeding from drain
21	Male	42	Pancreatic carcinoma	Pancreaticoduodenectomy	46	Haematemesis/melena
22	Male	45	Splenomegaly	Splenectomy	9	Bleeding from drain
23	Male	56	Bile duct stone	Choledocholithotomy	26	Bleeding from drain
24	Female	62	Gastric carcinoma	Gastrectomy	65	Hematochezia/melena
25	Male	76	Gastric carcinoma	Gastrectomy	29	Haematemesis/melena
26	Male	40	Mesenteric torsion	Partial intestinal resection	15	Haematemesis/melena

one patient with positive angiographic finding, because the bleeding vessels were capillaries and could not be superselected. This patient underwent a second surgery after angiography immediately, and recovered after second surgery. However, there were five patients without positive angiographic findings in our study. Among these five patients, one died of rebleeding after blind embolization; one recovered after conservative treatment; and the other three also recovered after a second operation. In the surgical procedure, we found that the bleeding vessels were the splenic vein, portal vein behind the gastrointestinal anastomotic stoma, and left gastro-omental vein, respectively.

Technical success was achieved in 21 (95%) of 22 patients (20 patients with positive angiographic findings and one without positive angiographic findings). Clinical success was achieved in 18 (82%) of 22 patients. Three patients were unsuccessfully treated, including two with rebleeding after embolization, and one with rebleeding after blind embolization.

Postembolization complications such as intestinal ischemia and liver infarction did not occur in any patients during the follow-up period. Three patients (two with positive angiographic findings and one without positive angiographic findings) died of rebleeding after embolization.

## DISCUSSION

Postoperative gastrointestinal hemorrhage is a life-threatening complication that occurs after abdominal surgery,

particularly in the case of pancreaticoduodenectomy. The incidence of postoperative gastrointestinal hemorrhage after abdominal surgery is not high (0.4%–18%)<sup>[1–4]</sup>. Early hemorrhagic complications occur during the first 24 h postoperatively, and are usually caused by intraoperative technical failure, such as improper ligation of vessels in the operative area, and damages to small vessels during lymph node dissection. Delayed postoperative hemorrhage has a different pathophysiology of bleeding from early postoperative hemorrhage, and is complicated with intra-abdominal lesions such as marginal ulcer, anastomotic leakage, intra-abdominal abscess, and sepsis. In this study, the interval from surgery to bleeding ranged from 3 to 65 d, and was > 5 d for the majority of patients. The intra-abdominal complications such as pancreatic juice leakage, intestinal juice leakage, and intra-abdominal abscess were the main causes of gastrointestinal bleeding in our study. In order to reduce the rate of postoperative gastrointestinal hemorrhage after abdominal surgery, we must decrease abdominal surgery complications, such as stomal leak, marginal ulcer, and abscess.

Early diagnosis and prompt treatment are necessary to decrease the mortality of patients with postoperative hemorrhage after abdominal surgery. Endoscopy is usually served as the first-line diagnostic procedure. However, exact diagnosis via urgent gastrointestinal endoscopy can be severely impaired by excessive blood and clots in the gastrointestinal tract<sup>[20,21]</sup>. computed tomography angiography, Doppler ultrasound, and radionuclide scanning can also be used in the diagnosis of postoperative hemorrhage<sup>[5,22,23]</sup>. Compared with these diagnosis

**Table 2** Angiographic findings and clinical results of patients with postoperative hemorrhage after abdominal surgery

Case	Angiography finding	Embolized artery	Embolic agent	Clinical results
1	Negative	None	None	Conservative treatment and clinical success
2	Negative	None	None	Repeat surgery and clinical success
3	Negative	None	None	Repeat surgery and clinical success
4	Negative	None	None	Repeat surgery and clinical success
5	Extravasation	None	None	Conservative treatment and clinical success
6	Extravasation	Inferior pancreaticoduodenal artery	Microcoil	Clinical success
7	Extravasation	Jejunal artery	Microcoil	Clinical success
8	Extravasation	Jejunal artery	Microcoil	Clinical success
9	Extravasation	Inferior pancreaticoduodenal artery	Microcoil	Clinical success
10	Extravasation	Great pancreatic artery	Gelfoam	Clinical success
11	Negative	Right hepatic artery and gastroduodenal artery	Gelfoam	Die of rebleeding
12	Extravasation	Ileocolic artery	Microcoil	Clinical success
13	Pseudoaneurysm	Gastroduodenal artery	Microcoil	Clinical success
14	Extravasation	Gastroduodenal artery	Microcoil	Clinical success
15	Pseudoaneurysm	superior rectal artery	Microcoil	Clinical success
16	Pseudoaneurysm	Gastroduodenal artery	Microcoil	Clinical success
17	Fusiform aneurysm	Gastroduodenal artery	Microcoil	Clinical success
18	Pseudoaneurysm	Common hepatic artery	Microcoil	Clinical success
19	Extravasation	Gastroduodenal artery	Microcoil	Die of rebleeding
20	Pseudoaneurysm	Gastroduodenal artery	Microcoil	Die of rebleeding
21	Fusiform aneurysm	Proper hepatic artery	Microcoil + gelfoam	Clinical success
22	Pseudoaneurysm	Splenic artery and Right gastroepiploic artery	Microcoil + gelfoam	Clinical success
23	Pseudoaneurysm	Right hepatic artery	Microcoil + gelfoam	Clinical success
24	Pseudoaneurysm	Inferior pancreaticoduodenal artery	Microcoil + gelfoam	Clinical success
25	Fusiform aneurysm	Gastroduodenal artery	Microcoil + gelfoam	Clinical success
26	Pseudoaneurysm	Jejunal artery	Microcoil + gelfoam	Clinical success

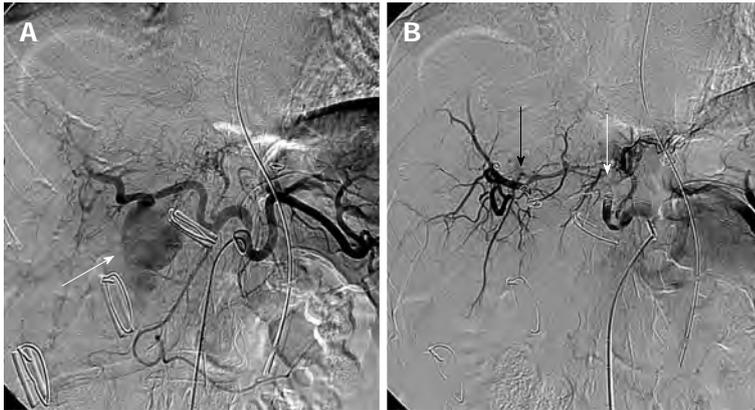


**Figure 1** A 35-year-old man with massive upper gastrointestinal bleeding after gastrectomy of gastric carcinoma. Selective superior mesenteric arteriography showed active arterial contrast extravasation (white arrow) from the jejunal artery.

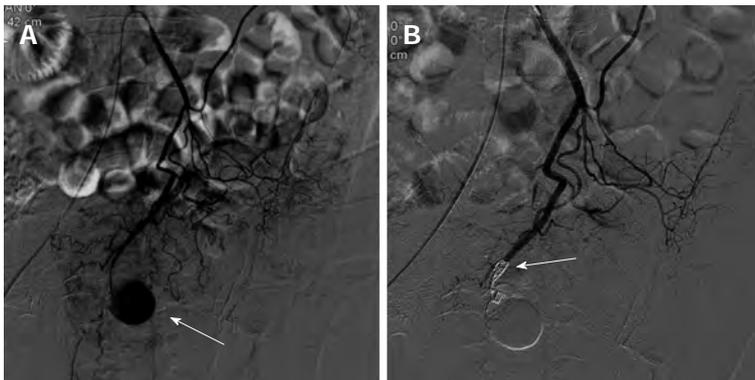
methods, angiography is quicker, safer, and more accurate to localize the bleeding points. In addition, angiography allows immediate embolization to stop gastrointestinal hemorrhage. Angiographic findings of postoperative gastrointestinal hemorrhage differ slightly from those of gastrointestinal hemorrhage without surgery. Positive angiographic findings of gastrointestinal hemorrhage without surgery mainly included extravasation of contrast medium, tumor staining, and vascular malformation. Charbonnet *et al*<sup>[24]</sup> reported that angiography had a positive angiographic rate of 31% in all consecutive patients without abdominal surgery. Kim *et al*<sup>[19]</sup> reported a positive angiographic rate of 79% in patients after abdominal surgery. However, positive angiographic find-

ings of postoperative gastrointestinal hemorrhage mainly included extravasation of contrast medium and pseudoaneurysms. In our study, the positive findings were 81% (21 of 26 patients), and the rate of positive findings was higher than that of gastrointestinal hemorrhage without abdominal surgery, and was similar to that of gastrointestinal hemorrhage with surgery. Therefore, angiography should be the first-choice option for postoperative gastrointestinal hemorrhage after abdominal surgery, especially for patients with hemodynamic instability and poor general conditions. If celiac angiography fails to identify a source of bleeding, superselective angiography near the surgical field should be performed. However, angiography also has some limitations. For example, the result of angiography could be negative because the gastrointestinal bleeding is often intermittent or directly comes from veins or has been controlled by vasoactive agents. In this study, angiography demonstrated bleeding points in 21 of 26 patients. Reoperation was performed in three patients with negative angiographic findings. We found that the bleeding vessels were the left gastro-omental vein, portal vein behind the anastomotic stoma, and splenic vein.

Reoperation to control postoperative bleeding is the traditional approach to manage gastrointestinal hemorrhage after abdominal surgery. However, emergency surgical exploration has been reported to be associated with a mortality rate of as high as 64% in high-risk patients with hemodynamic instability and poor general conditions<sup>[25,26]</sup>. In addition, the surgical approach is often difficult or even unsuccessful in patients with postoperative hemorrhage, especially those with two or more previous abdominal operations, due to the anatomical inaccessibili-



**Figure 2** A 56-year-old man presented with massive bleeding from surgical drains after choledocholithotomy due to common bile duct stone. A: Selective celiac arteriography showed a pseudoaneurysm (arrow) arising from the right hepatic artery; B: Selective celiac arteriography after embolization with microcoils and gelfoam demonstrated disappearance of the pseudoaneurysm. Embolic agents were inserted proximally (white arrow) and distally (black arrow) to the origin of the pseudoaneurysm.



**Figure 3** A 61-year-old man presented with massive bleeding from surgical drains 3 d after excision of the intra-abdominal abscess. A: Selective inferior mesenteric arteriography shows a pseudoaneurysm (arrow) arising from the superior rectal artery; B: Selective inferior mesenteric arteriography after embolization with microcoils demonstrated complete occlusion (arrow) of the distal end of the superior rectal artery.

ty of the arteries, postoperative adhesions, and inflammatory reaction. Endoscopy is another approach to manage postoperative hemorrhage<sup>[1-4,27-32]</sup>. However, emergency endoscopy for postoperative hemorrhage may be difficult owing to excessive blood and clots in the gastrointestinal tract, and inaccessibility of the bleeding sites in the small intestine.

Transarterial embolization is effective for postoperative hemorrhage, especially in patients with hemodynamic instability and poor general condition. However, the safety and clinical results of embolization have not been assessed in a large patient group. Beyer *et al*<sup>[5]</sup> reported that embolization had a success rate of 100% in nine patients with delayed hemorrhage after pancreaticoduodenectomy. Miyamoto *et al*<sup>[33]</sup> demonstrated a success rate of 80% with superselective embolization in 10 patients with massive upper gastrointestinal hemorrhage after upper abdominal surgery. Compared with these studies, the clinical success rate (82%) of the present study was similar. However, the technical success rate in this study was 95% because we used high-resolution digital angiography and microcatheterization. Three patients died during the follow-up period. The cause of death was rebleeding after embolization, including one blind embolization.

The clinical results of blind embolization (defined as embolization without positive angiography) are controversial. Morris *et al*<sup>[34]</sup> found that blind embolization of the left gastric artery was effective in preventing rebleeding when an active bleeding site was localized by endoscopy. Kim *et al*<sup>[19]</sup> successfully treated four patients

with blind embolization after an active bleeding site was identified by endoscopy or scintigraphy, or was suspicious on angiography. In our study, blind embolization was performed only in one patient after the bleeding site was localized by endoscopy. However, the patient died of rebleeding 3 d after the interventional procedure.

The most serious complications of postembolization are liver infarction and irreversible bowel ischemia. However, the liver can tolerate considerable arterial embolization without significant liver infarction, because the liver has a dual blood supply by the hepatic artery and portal vein, and the hepatic artery has abundant collateral pathways. Arterial embolization in the upper gastrointestinal tract above the ligament of Treitz is generally considered safe because of the rich collateral supply to the stomach and duodenum<sup>[34]</sup>. In contrast to the upper gastrointestinal tract, the lower gastrointestinal tract does not have a rich collateral artery, and is susceptible to embolization-induced ischemia. However, significant ischemia may be avoided if the embolic agent is delivered precisely to the bleeding sites. In our study, postembolization complications such as liver infarction and bowel ischemia were not encountered. Therefore, we believe that transarterial embolization is a safe method to treat postoperative gastrointestinal hemorrhage.

In conclusion, we retrospectively analyzed the angiographic findings and clinical outcomes of transarterial angiography and embolization in 26 patients with postoperative hemorrhage after abdominal surgery. Angiography was found to be a sensitive approach to detect the

bleeding site, especially for patients with postoperative gastrointestinal hemorrhage. Transarterial embolization is an effective and safe method for the treatment of postoperative hemorrhage.

## COMMENTS

### Background

Gastrointestinal hemorrhage after abdominal surgery is an unusual complication. However, when it occurs, it can cause hemorrhagic shock that has a fatal outcome. This complication may occur as a result of anastomotic leakage, localized infection, or intraoperative arterial injury. Traditionally, reoperation is regarded as the first-line therapy. However, its usage is largely limited by the poor condition of patients with postoperative gastrointestinal hemorrhage and the difficulty in indentifying the bleeding sites during surgery. With advances in technology, a newer and less-invasive technique, transcatheter arterial embolization, has been developed and is reported to be safe and effective, especially in high-risk surgical patients.

### Research frontiers

For postoperative gastrointestinal hemorrhage, the first important thing is to localize the bleeding site. Transarterial angiography is considered to be a better tool than endoscopy or noninvasive diagnostic imaging examinations such as computed tomography angiography and ultrasound. Transarterial access not only can provide diagnostic information but also has the advantage of intra-arterial embolization of the bleeding site simultaneously.

### Innovations and breakthroughs

The surgical approach to control postoperative bleeding is often difficult or even unsuccessful in patients with postoperative hemorrhage, especially those with two or more previous abdominal operations, due to the anatomical inaccessibility of the arteries, postoperative adhesions, and inflammatory reaction. Therefore, its mortality rate was as high as 64% in high-risk patients with hemodynamic instability and poor general condition. However, with advances in techniques and microcatheters, transcatheter arterial embolization was performed to control postoperative bleeding. In this retrospective study, the technical success rate was 95%, and the clinical success rate was 82%. There were no procedure-related complications.

### Applications

The results of the present study suggest that transcatheter arterial embolization is a safe and effective treatment for massive gastrointestinal hemorrhage following abdominal surgery.

### Peer review

This was a good descriptive study in which the authors evaluated the clinical results of angiography and embolization for massive gastrointestinal hemorrhage after abdominal surgery. The results are interesting and suggest that transcatheter arterial embolization is a safe and effective treatment for massive gastrointestinal hemorrhage following abdominal surgery, especially for patients with hemodynamic instability and poor general condition.

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## Overexpression of nuclear $\beta$ -catenin in rectal adenocarcinoma is associated with radioresistance

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clinicopathological characteristics was also analyzed. Univariate and logistic multivariate regression analyses were adopted to determine the independent factors of radioresistance.

**RESULTS:** Nuclear  $\beta$ -catenin overexpression was more evident in radioresistant rectal adenocarcinoma than in radiosensitive rectal adenocarcinoma (57.6% vs 16.7%,  $P < 0.001$ ). Nuclear  $\beta$ -catenin was overexpressed in favor of poor TRG ( $\leq 2$ ), whereas membrane  $\beta$ -catenin was expressed in favor of good TRG ( $\geq 3$ ). Nuclear  $\beta$ -catenin expression in tumor cell differentiation ( $P = 0.018$ ), lymph node metastasis ( $P = 0.022$ ), and TRG ( $P < 0.001$ ) showed significant differences. Univariate analyses demonstrated that radioresistance is associated with nuclear  $\beta$ -catenin overexpression ( $P < 0.001$ ). In addition, logistic multivariate regression analysis indicated that only three factors, namely, tumor size ( $P < 0.001$ ), tumor cell differentiation ( $P < 0.001$ ), and nuclear  $\beta$ -catenin overexpression ( $P < 0.001$ ), are associated with radioresistance. By using radioresistance as a prediction target, nuclear  $\beta$ -catenin-based prediction alone achieved 83% accuracy, 65% sensitivity, and 88% specificity.

**CONCLUSION:** Nuclear  $\beta$ -catenin overexpression may be a valuable candidate to predict the response of rectal adenocarcinoma to preoperative radiotherapy.

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**Key words:**  $\beta$ -catenin; Rectal cancer; Preoperative radiotherapy; Radioresistance; Colorectal cancer

**Core tip:** In this paper we investigated the relationship between overexpression of nuclear  $\beta$ -catenin in rectal adenocarcinoma and radioresistance. We first confirmed that nuclear  $\beta$ -catenin overexpression in rectal adenocarcinoma is associated with radioresistance. Most importantly, we found that nuclear  $\beta$ -catenin-based prediction achieved a 83% accuracy, 65% sen-

### Abstract

**AIM:** To investigate the association between nuclear  $\beta$ -catenin overexpression in rectal adenocarcinoma and radioresistance.

**METHODS:** A retrospective analysis was conducted. The analysis involved 136 patients with locally advanced rectal adenocarcinoma who underwent short-course preoperative radiotherapy and radical resection. The expression of  $\beta$ -catenin in both pretreatment biopsy specimens and resected primary tumor tissues was examined by immunohistochemistry. The correlation of  $\beta$ -catenin expression with radioresistance was evaluated using the tumor regression grading (TRG) system. The relationship between  $\beta$ -catenin expression and

sitivity and 88% specificity for radioresistance. We provided a novel possible molecular mechanism to explain the radioresistance in rectal adenocarcinoma and thus may provide a new therapeutic target for enhancing radiosensitivity.

Wang L, Zhang XM, Li Z, Liu XJ, Chai J, Zhang GY, Cheng YF. Overexpression of nuclear  $\beta$ -catenin in rectal adenocarcinoma is associated with radioresistance. *World J Gastroenterol* 2013; 19(40): 6876-6882 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6876.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6876>

## INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy and the fourth most frequent cause of cancer deaths worldwide. Surgery is the predominant therapy for rectal cancer. However, rectal cancer treatment with surgery alone is insufficient because of the high incidence of local recurrence. During the past decades, preoperative radiotherapy and surgery have been increasingly used together for locally advanced rectal cancer to reduce local treatment failure<sup>[1-4]</sup>. However, many rectal cancer patients are resistant to preoperative radiotherapy because of the heterogeneity of treatment response. Therefore, predicting neoadjuvant radiotherapy response may allow individualization and more rational selection of patients that will most likely benefit from this therapy. A growing number of studies have investigated molecular markers for the prediction of tumor response to radiotherapy. XIAP<sup>[5]</sup>, human phosphatidylethanolamine-binding protein 4<sup>[6]</sup>, and epidermal growth factor receptor<sup>[7]</sup> are the molecular markers associated with tumor response to radiotherapy in rectal cancer.

$\beta$ -catenin, a component of the Wntless/Wnt signaling pathway, can activate target genes linked to the *APC* gene in CRC. The localization of  $\beta$ -catenin is related to its function in cancer growth<sup>[8]</sup>.  $\beta$ -catenin in the cytoplasm and membrane binds with the intracellular domain of E-cadherin, a cell-to-cell adhesion molecule, and maintains normal tissue architecture. In the presence of a Wnt signal,  $\beta$ -catenin translocates to the nucleus and interacts with the lymphoid enhancing factor/T-cell factor to promote the transcription of several target genes involved in cell proliferation<sup>[9]</sup>.

A previous study indicated that Wnt/ $\beta$ -catenin mediates the radiation resistance of mouse mammary progenitor cells and Sca1+ progenitors in an immortalized mammary gland cell line<sup>[10,11]</sup>. Thus, an active interaction probably exists between the radiotherapy response of cancer cells and Wnt/ $\beta$ -catenin signal pathway. However, no direct evidence about the correlation between nuclear  $\beta$ -catenin expression in rectal cancer and radiotherapy sensitivity has been reported to date. In this study, we enrolled 136 rectal adenocarcinoma patients treated with

fractionated preoperative radiotherapy and radical resection. We investigated the expression of nuclear  $\beta$ -catenin in rectal adenocarcinoma using biopsy specimens and resected primary tumor tissues to determine whether nuclear  $\beta$ -catenin expression can be a predictive marker of tumor response to preoperative radiotherapy.

## MATERIALS AND METHODS

### Study population

The study cohort comprised 136 rectal cancer patients who had undergone consecutive preoperative radiotherapy (25 Gy in five fractions for 1 wk) and radical surgery at Qilu Hospital and Shandong Tumor Hospital and Institute from November 2008 to June 2011. The subjects included 98 men and 38 women with a mean age of 64 years (range: 35 years to 78 years). First, rectal cancer was diagnosed by contrast computed tomography (CT) and colonoscopy. Afterward, archival paraffin-embedded biopsy tumor specimens and post-surgery resected tumor tissues were histologically identified as rectal adenocarcinoma.

The inclusion criteria were as follows: (1) patients histologically diagnosed with rectal adenocarcinoma; and (2) rectal cancer clinically diagnosed as stage III. The exclusion criteria were as follows: (1) hereditary nonpolyposis CRC patients and patients with familial adenomatous polyposis; and (2) patients with distant metastasis. All 136 patients met the inclusion criteria and were included in this study. However, 11 cases with distant metastasis and 1 case with familial adenomatous polyposis were excluded.

All cancers were clinically diagnosed as stage III with no distant metastasis. Tumor stage was based on the American Joint Committee on Cancer (AJCC) TNM staging system (7<sup>th</sup> edition, 2009). The TNM stages of the patients in this cohort were as follows: 28 patients with stage IIIA, 46 patients with stage IIIB, and 62 patients with stage IIIC.

All patients received preoperative radiotherapy and underwent Miles' operation. The distance from distal margin of rectal cancer to anal edge was less than 7 cm in all patients. The interval between preoperative radiotherapy and surgery was 10 d to 14 d.

### Data collection and design of the study

This study was approved by the Ethics Committees of Qilu Hospital, Shandong University, and Shandong Tumor Hospital and Institute. Informed consent was obtained from each patient. Clinical information related to diagnostic procedures and tumor characteristics was collected from medical records. Clinical and pathological data, including sex, age, tumor size, tumor invasion depth, tumor cell differentiation, and lymph node metastasis, were collected. Firstly, the association between expression of nuclear  $\beta$ -catenin in biopsy specimens and respective resected tumor tissues was retrospectively analyzed. Meanwhile, tumor regression grading (TRG) of rectal ad-

enocarcinoma after radiotherapy was evaluated. Then, the relationship between nuclear  $\beta$ -catenin expression in rectal adenocarcinoma and clinicopathological characteristics was retrospectively analyzed. Finally, univariate analysis and logistic multivariate regression analysis were adopted to discriminate independent factors of radioresistance.

### Immunohistochemistry

Biopsy specimens obtained through colonoscopy and resected tumor tissues collected after surgery were embedded in paraffin and cut into sections for immunohistochemical staining using the streptavidin peroxidase complex method. Tissue sections (4  $\mu$ m thick) were deparaffinized and microwaved for 15 min, incubated twice in 10 mmol/L citrate buffer at 100 °C to retrieve the antigens, and then incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 min to quench endogenous peroxidase. Nonspecific binding of antibodies was inhibited by incubation in 5% normal goat serum for 20 min in a humid chamber. The tissue sections were then incubated with mouse monoclonal anti- $\beta$ -catenin antibodies (diluted 1:50, mouse IgG1; Cell Signaling Technology, Boston, United States) overnight at 4 °C. The tissue sections were washed three times with PBS and then incubated with biotinylated goat antimouse IgG for 30 min at room temperature. After washing, the slides were incubated in a streptavidin-peroxidase complex for 20 min at 37 °C, washed three times, visualized using 3,3'-diaminobenzidine, and then counterstained with hematoxylin. Sections that were stained without the primary antibodies served as the negative control.  $\beta$ -catenin expression is defined as brown colored staining on the membrane or in the cytoplasm and nucleus. For immunohistochemical assessment, six slides were randomly selected and observed at a magnification of  $\times$  200 by two pathologists. The immunostained slides were scored as previously described<sup>[12]</sup>. In brief, the immunostained slides were scored using the sum of the signal intensity (0 = no expression; 1 = weak expression; 2 = moderate expression; 3 = strong expression) and the percentage of positive cells (% tumor cells: 0 = 0%; 1 = 1% to 25%; 2 = 26% to 50%; 3 = 51% to 75%; and 4 = 76% to 100%). Nuclear  $\beta$ -catenin immunoreactivity at the invasive front was considered positive if moderate or strong expression was observed in the nuclei. Nuclear  $\beta$ -catenin overexpression was considered positive when the expression was observed in > 50% of the tumor cells.

### TRG

The response of rectal cancer to preoperative radiotherapy was evaluated in the hematoxylin and eosin-stained slides using the TRG system as previously described<sup>[13]</sup>. The characteristics of each grade were as follows: TRG 0, no regression; TRG 1, dominant tumor mass with obvious fibrosis in 25% or less of the tumor mass; TRG 2, dominant tumor mass with obvious fibrosis in 26 to 50% of the tumor mass; TRG 3, dominant fibrosis outgrowing the tumor mass; and TRG 4, no viable tumor cells

(only a fibrotic mass).

### Statistical analysis

Statistical analysis was performed using SPSS16.0.  $\chi^2$  test and Fisher exact test were used to analyze the correlation between nuclear  $\beta$ -catenin expression and clinicopathological characteristics. Univariate and logistic multivariate regression analyses were performed to determine the independent factors of TRG.  $P < 0.05$  was considered statistically significant.

## RESULTS

### $\beta$ -catenin expression in biopsy specimens and in respective resected tumor tissues

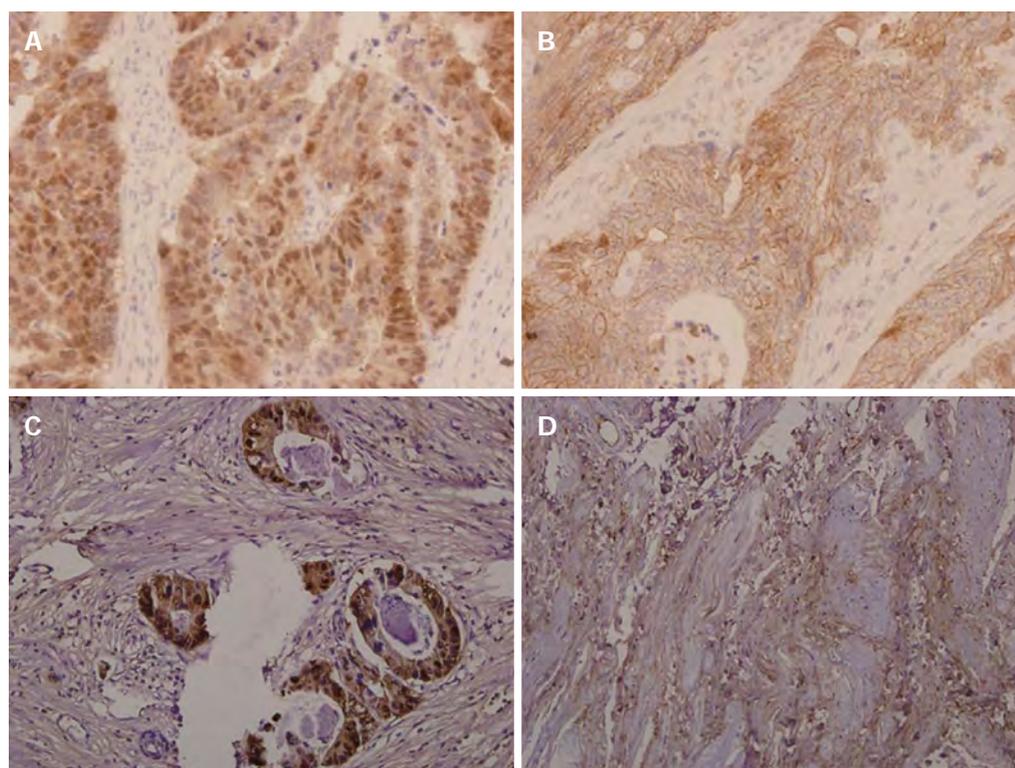
The expression of  $\beta$ -catenin in the biopsy and respective resected tumor tissues is presented in Figure 1.  $\beta$ -catenin expression was observed in all rectal cancer specimens.  $\beta$ -catenin was expressed mostly in a nuclear-associated staining pattern in patients who exhibited resistance to preoperative radiotherapy (Figure 1A). Conversely,  $\beta$ -catenin was expressed mostly in a membrane-associated staining pattern in patients who exhibited hyper-radio-sensitivity (Figure 1B). Nuclear  $\beta$ -catenin overexpression was observed in 68 cases who exhibited radioresistance (57.6%). However, this overexpression was only observed in three patients who exhibited radiosensitivity (16.7%). Therefore, nuclear  $\beta$ -catenin overexpression is more evident in radioresistant patients than in radiosensitive patients ( $P < 0.001$ , Table 1). After preoperative radiotherapy, nuclear  $\beta$ -catenin was overexpressed in favor of poor TRG ( $\leq 2$ ) (Figure 1C), whereas membrane  $\beta$ -catenin was overexpressed in favor of good TRG ( $\geq 3$ ) (Figure 1D). This result suggests that nuclear  $\beta$ -catenin overexpression is a predictive marker of radioresistance.

### Relationship between nuclear $\beta$ -catenin expression in rectal adenocarcinoma and clinicopathological characteristics

$\chi^2$  test was performed to investigate the relationship between nuclear  $\beta$ -catenin expression and clinicopathological features. The correlations between nuclear  $\beta$ -catenin expression level and clinicopathological variables are shown in Table 1. Nuclear  $\beta$ -catenin expression in tumor cell differentiation ( $P = 0.018$ ), lymph node metastasis ( $P = 0.022$ ), and TGR ( $P < 0.001$ ) showed significant differences.

### Predictive value of nuclear $\beta$ -catenin overexpression for radioresistance

Clinicopathological characteristics were divided into two groups according to tumor response to radiotherapy to confirm the predictive value of nuclear  $\beta$ -catenin overexpression as a biomarker of radioresistance. Univariate and multivariate analyses were performed to determine the factors related to radioresistance. Univariate analysis results (Table 2) demonstrated that radioresistance was associated with tumor size ( $P < 0.001$ ), tumor cell differ-



**Figure 1**  $\beta$ -catenin expression in biopsy specimens and resected tumor tissues ( $\times 200$ ). A: Nuclear  $\beta$ -catenin was overexpressed in biopsy specimens from patients who exhibited resistance to preoperative radiotherapy; B:  $\beta$ -catenin presented mostly in a membrane-associated staining pattern in biopsy specimens from patients who exhibited hyper-radiosensitivity; C: Nuclear  $\beta$ -catenin was overexpressed in specimens with poor tumor regression grading (TRG) ( $\leq 2$ ); D:  $\beta$ -catenin presented mostly in a membrane-associated staining pattern in specimens with good TRG ( $\geq 3$ ).

**Table 1** Correlation of nuclear  $\beta$ -catenin expression in rectal adenocarcinoma with clinicopathologic characteristics

Characteristics	n	Nuclear $\beta$ -catenin expression		P value <sup>1</sup>
		$\leq 50\%$	$> 50\%$	
Gender				
Male	108	54	54	0.312
Female	28	11	17	
Age (yr)				
$\leq 55$	19	11	8	0.342
$> 55$	117	54	63	
Tumor size (cm)				
$\leq 5$	78	38	40	0.802
$> 5$	58	27	31	
Tumor cell differentiation				
Well/moderately differentiated	98	53	45	0.018
Poorly differentiated	38	12	26	
Tumor invasion depth				
T1/T2	23	15	8	0.066
T3/T4	113	50	63	
Lymph node metastasis				
N1	74	42	32	0.022
N2	62	23	39	
TRG				
TRG0-2	118	50	68	$< 0.001$
TRG3-4	18	15	3	

<sup>1</sup>Value is the result of  $\chi^2$  test in the same factor, and  $P < 0.05$  is considered significant statistically. TRG: Tumor regression grading.

entiation ( $P < 0.001$ ), tumor invasion depth ( $P = 0.027$ ), and nuclear  $\beta$ -catenin expression ( $P < 0.001$ ). However, logistic multivariate regression analysis retained only three factors in the model, namely, tumor size ( $P < 0.001$ ), tumor cell differentiation ( $P < 0.001$ ), and nuclear  $\beta$ -catenin overexpression ( $P < 0.001$ ). By using radioresistance as a prediction target, nuclear  $\beta$ -catenin-based prediction alone achieved 83% accuracy, 65% sensitivity, and 88% specificity (Table 3).

## DISCUSSION

In the present study, the expression of  $\beta$ -catenin in 136 rectal adenocarcinoma patients was detected and its relationship with radioresistance was investigated. Our data demonstrated that overexpression of nuclear  $\beta$ -catenin is associated with radioresistance in rectal adenocarcinoma. To the best of our knowledge, few studies have been done to investigate the prognostic value of nuclear  $\beta$ -catenin overexpression for radioresistance in rectal cancer. The clinical significance of the current study is that we might provide promising biomarkers to predict sensitivity of rectal cancer to radiotherapy.

Preoperative radiotherapy has become a standard treatment for locally advanced rectal cancer. Obtaining an accurate prediction of cancer cell response to radiothera-

**Table 2** Univariate and multivariate regression analyses of the association of clinical features with radiotherapy response of rectal adenocarcinoma *n* (%)

Clinicopathological factors	Univariate analysis		Multivariate analysis	
	Good response <sup>1</sup>	<i>P</i> value	OR	<i>P</i> value
Gender				
Male	15 (13.8)	0.697		NS
Female	3 (10.7)			
Age (yr)				
≤ 55	5 (26.3)	0.129		NS
> 55	13 (11.1)			
Tumor size (cm)				
≤ 5	16 (20.5)	< 0.001	5.058	< 0.001
> 5	2 (3.5)			
Tumor cell differentiation				
Well/moderately differentiated	4 (4.1)	< 0.001	4.692	< 0.001
Poorly differentiated	14 (36.8)			
Tumor invasion depth				
T1/T2	7 (30.4)	0.027	1.143	0.053
T3/T4	11 (9.7)			
Nuclear $\beta$ -catenin expression				
≤ 50%	15 (23.1)	< 0.001	6.375	< 0.001
> 50%	3 (4.2)			

<sup>1</sup>Tumor regression grading  $\geq$  3. NS: Not selected.

**Table 3** Predictive value of nuclear  $\beta$ -catenin as a marker of radioresistance in 136 patients of rectal adenocarcinoma

Nuclear $\beta$ -catenin expression	Good response	Poor response
≤ 50%	15%	50%
> 50%	3%	68%
Accuracy		83%
Sensitivity		65%
Specificity	88%	
Positive predictive value		79%
Negative predictive value	48%	

py to determine if a patient would benefit from adjuvant therapy is challenging<sup>[14]</sup>. Previous studies indicated that molecules involved in signaling pathways are crucial to the sensitivity of cancer cells to radiotherapy<sup>[15,16]</sup>. Numerous studies have investigated biomarkers to predict rectal cancer sensitivity to radiotherapy<sup>[17-19]</sup>. However, only a few scholars have focused on the Wnt/ $\beta$ -catenin signaling pathway, which is vital in the progression of rectal cancer.

$\beta$ -catenin is a component of the Wntless/Wnt signaling pathway. Previous studies have demonstrated that  $\beta$ -catenin is associated with the sensitivity of some cancers to radiotherapy. Kim *et al.*<sup>[20]</sup> found that glioblastoma (GBM) cell lines enriched with cells positive for active  $\beta$ -catenin are increased by *in vitro* radiation treatment. This finding suggests that the radiation resistance of GBM is partly mediated by the activation of stem cell-associated pathways, including Wnt. Watson *et al.*<sup>[21]</sup> found that the expression of a kinase dead mutant GSK3 $\beta$  endows Panc1 and BxPC3 pancreatic cancer cells with radioresistance.  $\beta$ -catenin silencing results in radiosensitization, whereas a nondegradable  $\beta$ -catenin construct induces radioresistance. These data support the

hypothesis that GSK3 $\beta$  modulates the cellular response to radiation in a  $\beta$ -catenin-dependent mechanism. In the current study, only stage III rectal adenocarcinoma patients who accepted preoperative radiotherapy were included to eliminate the effects of different therapeutic methods, such as concurrent chemoradiotherapy. The results indicated that nuclear  $\beta$ -catenin overexpression is more evident in tumor biopsy specimens from patients who exhibited radioresistance. We also found that tumor tissues were relatively intact when nuclear  $\beta$ -catenin was overexpressed in tumor cells after preoperative radiotherapy. These immunohistochemical data indicated that the accumulation of nuclear  $\beta$ -catenin in tumor cells is critical in the radioresistance of rectal adenocarcinoma.

Regarding the correlation between nuclear  $\beta$ -catenin expression and clinicopathological characteristics, previous investigators reported contradictory results. Zhang *et al.*<sup>[22]</sup> believed that nuclear  $\beta$ -catenin accumulation is related to tumor stage and/or metastasis. However, correlations between nuclear  $\beta$ -catenin and pertinent clinicopathological variables were not observed in Baldus's study<sup>[23]</sup>. Our data demonstrated that nuclear  $\beta$ -catenin overexpression is related to low tumor cell differentiation and lymph node metastasis. Hyper-radiosensitivity is associated with low tumor cell differentiation. In the current study, a relationship was found between nuclear  $\beta$ -catenin overexpression and low tumor cell differentiation. However, tumor tissues in which nuclear  $\beta$ -catenin was overexpressed exhibited resistance to preoperative radiotherapy. This result suggests that nuclear  $\beta$ -catenin overexpression is a potential mechanism by which rectal adenocarcinoma cells avoid the destructive effect of radiotherapy.

Univariate and multivariate analyses were used to determine the factors related to radioresistance and thus confirm the predictive value of nuclear  $\beta$ -catenin overex-

pression for radioresistance. Univariate analysis demonstrated that nuclear  $\beta$ -catenin overexpression is associated with tumor response to radiotherapy. This finding coincides with the immunohistochemical result. A multivariate logistic regression analysis model was constructed to distinguish the independent factors for radioresistance and obtain a more precise estimate of the effect of nuclear  $\beta$ -catenin overexpression on tumor response to radiotherapy. As shown in Table 2, tumor response to radiotherapy is associated with nuclear  $\beta$ -catenin overexpression. This result suggests that nuclear  $\beta$ -catenin overexpression may affect radioresistance independently. When radioresistance was used as a prediction target in this study, nuclear  $\beta$ -catenin overexpression-based prediction alone achieved 83% accuracy, 65% sensitivity, and 88% specificity. These data confirmed that our conclusion is consistent with previous findings and suggested that nuclear  $\beta$ -catenin overexpression in rectal adenocarcinoma is a useful marker of radioresistance. Prediction of rectal cancer patient response is critical in tailoring preoperative radiotherapy to individuals. In addition, the search for predictive markers of radiotherapy is similar to the development of targeted therapy to some extent. We believe that  $\beta$ -catenin is a potential target for reducing radioresistance in rectal adenocarcinoma. In the future,  $\beta$ -catenin-targeting molecular drugs and radiation therapy may be used in combination to improve the efficacy of radiotherapy for rectal adenocarcinoma.

There are some potential limitations of this study. First, this is a retrospective study, and the confounding effects associated with a design of this kind are surely present. Second, only those patients who accepted preoperative radiotherapy were included in order to study the prognostic value of nuclear  $\beta$ -catenin overexpression for radioresistance in this study. Having only a small number of patients in the study also prevented us making further analysis about the correlation of nuclear  $\beta$ -catenin overexpression with clinical outcome. We believe that a larger prospective trial and long-term follow up study will allow us to confirm our conclusions.

In conclusion, nuclear  $\beta$ -catenin overexpression in rectal adenocarcinoma is associated with radioresistance. Both clinical and pathological data support our conclusion. Therefore, nuclear  $\beta$ -catenin overexpression in rectal adenocarcinoma can be used as a valuable predictor of radioresistance. However, these preliminary findings must be verified in a larger, prospective, controlled clinical study and in a subsequent experimental study.

## COMMENTS

### Background

Radiotherapy is a major treatment for rectal cancer. During previous decades, preoperative radiotherapy has increasingly been used together with surgery for locally advanced rectal cancer in order to reduce local treatment failures. However, many rectal cancer patients are actually resistant to preoperative radiotherapy due to the heterogeneity of treatment response. Therefore, the ability to predict response for neoadjuvant radiotherapy may allow individualization and more rational selection of patients that will most likely benefit from this therapy.

### Research frontiers

A previous study indicated that Wnt/ $\beta$ -catenin mediates radiation resistance of mouse mammary progenitor cells and Sca1+ progenitors in an immortalized mammary gland cell line. Thus, there seems to be active interaction between the response to radiotherapy of cancer cells and the Wnt/ $\beta$ -catenin signal pathway. However, there is no direct evidence about the correlation between nuclear  $\beta$ -catenin expression in rectal cancer and radiotherapy sensitivity up to now.

### Innovations and breakthroughs

In this paper the authors investigated the relationship between overexpression of nuclear  $\beta$ -catenin in rectal adenocarcinoma and radioresistance. To the best of the knowledge, this is the first study that analyzes the relation between nuclear  $\beta$ -catenin overexpression in rectal adenocarcinoma and radioresistance. The authors provided a novel possible molecular mechanism to explain the radioresistance in rectal adenocarcinoma and thus may provide a new therapeutic target for enhancing radiosensitivity.

### Applications

By studying the relationship between overexpression of nuclear  $\beta$ -catenin in rectal adenocarcinoma and radioresistance, this study may provide a new therapeutic target for enhancing radiosensitivity for rectal adenocarcinoma.

### Terminology

$\beta$ -catenin is a component of the Wntless/Wnt signaling pathway and can activate target genes linking with the APC gene in colorectal cancer. Its localization relates to its function in cancer growth.  $\beta$ -catenin in the cytoplasm and membrane binds with the intracellular domain of E-cadherin, which is a cell-to-cell adhesion molecule, and to play a significant role in maintaining the normal tissue architecture.

### Peer review

The authors studied the clinical significance of  $\beta$ -catenin overexpression and radioresistance in patients with rectal adenocarcinoma. This paper is very interesting and the information is up to date. The study provided a novel possible molecular mechanism to explain radioresistance in rectal adenocarcinoma and thus may provide a new therapeutic target for enhancing radiosensitivity.

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## Expression and significance of cyclooxygenase-2 mRNA in benign and malignant ascites

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### Abstract

**AIM:** To investigate the mRNA expression of cyclooxygenase-2 (COX-2) in benign and malignant ascites, and to explore the difference in COX-2 mRNA expression among different diseases.

**METHODS:** A total of 36 samples were collected from the Fifth Affiliated Hospital of Sun Yat-Sen University and divided into two experimental groups: benign ascites ( $n = 21$ ) and malignant ascites ( $n = 15$ ). Benign ascites included cirrhotic ascites ( $n = 10$ ) and tuberculous ascites ( $n = 5$ ). Malignant ascites included oophoroma ( $n = 7$ ), cancer of colon ( $n = 5$ ), cancer of the liver ( $n = 6$ ), gastric cancer ( $n = 2$ ), and bladder carcinoma ( $n = 1$ ). The mRNA expression of COX-2 in ascites was examined with reverse transcriptase polymerase chain reaction (RT-PCR) technology, and the

positive rate of COX-2 mRNA was compared between different diseases.

**RESULTS:** The positive rate of COX-2 mRNA in malignant ascites was 42.9% (9/21), which was significantly higher than in benign ascites, 6.7% (1/15), difference being significant between these two groups ( $\chi^2 = 4.051$ ,  $P = 0.044$ ). The proportion of the positive rate in the malignant ascites was as follows: ovarian cancers 57.1% (4/7), colon cancer 40.0% (2/5), liver cancer 33.3% (2/6), gastric cancer 50.0% (1/2), and bladder cancer 0.00% (0/1). However, there was no significant difference in COX-2 mRNA expression among various tumors with malignant ascites ( $\chi^2 = 1.614$ ,  $P = 0.806$ ). Among the benign ascites, COX-2 mRNA levels were different between the tuberculous ascites (0/5) and cirrhotic ascites (1/10), but there was no significant difference ( $P = 1.000$ ).

**CONCLUSION:** COX-2 mRNA, detected by RT-PCR, is useful in the differential diagnosis of benign and malignant ascites, which also has potential value in the clinical diagnosis of tumors.

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**Key words:** Ascites; Cyclooxygenase-2 mRNA; Reverse transcriptase polymerase chain reaction; Malignant tumor

**Core tip:** Ascites is a common symptom caused by a variety of diseases, the differential diagnosis between benign ascites and malignant ascites is one of the most important issues in clinical practice. Cytologic examinations and ascites tumor markers can provide important evidence, but their sensitivity and specificity are far from satisfactory. Our study aimed to explore the difference in cyclooxygenase-2 (COX-2) mRNA expression among different diseases. Our research suggests that COX-2mRNA can be detected by reverse transcriptase polymerase chain reaction, but there are no significant differences in the expression of COX-2 mRNA among

various disease types with benign or malignant ascites.

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## INTRODUCTION

Ascites is a gastroenterological term for an accumulation of fluid in the peritoneal cavity, which might result from cirrhosis, tuberculous peritonitis or a malignant tumor.

The detection of exfoliated ascites cells is often used to identify these diseases in the clinic, but there is no economic, practical, or effective index with a high specificity. Therefore, it is important to discriminate benign from malignant ascites for clinical diagnosis and treatment<sup>[1,2]</sup>.

In recent years, cyclooxygenase-2 (COX-2) has been extensively studied as an inducible expression protein, and has been detected in various tumor tissues in epidemiologic and cytologic researches<sup>[3,4]</sup>, such as pancreatic cancer, colorectal carcinoma, non-small lung cancer and so on. Research has found that COX-2 expression is unregulated in precancerous lesions and preinvasive carcinoma and positively correlates with tumor invasion and lymphatic metastasis<sup>[5,6]</sup>. Therefore, increasing expression of COX-2 might occur in the early stages of the tumor and the detection of COX-2 level is helpful for early diagnosis.

However, there are only few studies regarding COX-2 expression in benign and malignant ascites. We employed reverse transcriptase polymerase chain reaction (RT-PCR) technology to detect the expression level of COX-2 in benign and malignant ascites, and analyzed the difference in mRNA expression of COX-2 among different diseases.

## MATERIALS AND METHODS

### Subjects

A total of 36 patients with ascites who underwent abdominocentesis at the Fifth Affiliated Hospital of Sun Yat-Sen University between August 2011 and March 2012 were selected. The subjects were divided into benign and malignant groups according to medical history, physical examination, B ultrasound, computed tomography (CT), pathology and the presence of exfoliated tumor cells. There were 15 patients with benign ascites, including nine males and six females, aged 43-75 years with an average age of  $62.5 \pm 1.8$  years; the patients consisted of 10 cases of cirrhosis and five cases of tuberculous peritonitis according to disease type. There were 21 patients with malignant ascites, including 11 males and 10 females, aged 41-79 years with an average age of  $58 \pm 2.3$  years; the

**Table 1** Primer sequences of cyclooxygenase-2 and glyceraldehyde-3-phosphate dehydrogenase genes

Primer	Primer sequence	Product size	
COX-2	Forward primer	5'-CTTGGGTGTCAAAGGTAA-3'	581 bp
	Reverse primer	5'-AGGGACTTGAGGAGGGTA-3'	
GAPDH	Forward primer	5'-GTGGGGCGCCAGGCACCA-3'	146 bp
	Reverse primer	5'-CTCCTATGTCACGCACATTC-3'	

COX-2: Cyclooxygenase-2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

patients consisted of seven cases of ovarian cancer, five cases of colon cancer, six cases of liver cancer, two cases of gastric cancer and one case of bladder cancer according to disease type.

The samples were centrifuged at 3000 r/min for 15 min immediately after collection from the patients, and the supernatant was removed. The pellet was stored in -80 °C refrigerator for total RNA extraction.

The sample collection was approved by the Ethics Committee of the hospital and all patients provided written informed consent.

### RT-PCR

**Primer design:** The gene sequences of the primers were designed with Primer Premier 5.0 software according to the literature<sup>[5,6]</sup> and mRNA sequences for human COX-2 reported by NCBI; GAPDH was selected as the positive control primer for RT-PCR and synthesized by the Shanghai ShengGong Biological Engineering Co., Ltd., China (Table 1).

**Total RNA extraction:** Total RNA was extracted from benign and malignant ascites with RNA extraction reagent (Trizol, Invitrogen, United States), and its content and purity were measured by ultraviolet spectrophotometry [ $1.9 < D(260)/D(280) < 2.1$ ].

**Synthesis of cDNA by reverse transcription:** Reverse transcription was performed to synthesize cDNA with the Avian Myeloblastosis Virus (AMV) Reverse Transcriptase Kit, which contained AMV reverse transcriptase and olig(dt)<sub>18</sub> primer. The RT-PCR reaction mixture of 10 μL contained 1 μL extracted total RNA, 2 μL MgCl<sub>2</sub>, 1 μL 10 × RNA PCR buffer, 3.75 μL RNase free dH<sub>2</sub>O, 1 μL dNTP mixture, 0.25 μL RNase inhibitor, 0.5 μL AMV reverse transcriptase and 0.5 μL Oligo dT-adaptor primer. The reaction conditions were set at 30 °C for 10 min, 50 °C for 30 min, 99 °C for 5 min and 5 °C for 5 min.

**PCR amplification:** After reverse transcription, cDNA was used as the template in PCR amplification with primers for COX-2 and glyceraldehyde-3-phosphate dehydro-

**Table 2** mRNA expression of cyclooxygenase-2 *n* (%)

Group	COX-2 mRNA expression		$\chi^2$	<i>P</i> value
	Positive	Negative		
In benign and malignant ascites <sup>1</sup>			4.051	0.044
Benign ascites	1 (6.7)	14 (93.3)		
Malignant ascites	9 (42.9)	12 (57.1)		
Among different disease types in benign group <sup>2</sup>				1.000
Cirrhosis with ascites	1 (6.7)	9 (60)		
Tuberculous ascites	0 (0.0)	5 (33.3)		
Among different disease types in malignant group <sup>3</sup>			1.614	0.806
Ovarian cancer	4 (19.0)	3 (14.3)		
Colon cancer	2 (9.5)	3 (14.3)		
Liver cancer	2 (9.5)	4 (19.0)		
Gastric cancer	1 (4.8)	1 (4.8)		
Bladder cancer	0 (0.0)	1 (4.8)		

<sup>1</sup>Comparison was made between groups using Yates' continuity correction;

<sup>2</sup>Comparison was made between groups using Fisher's exact probability

test; <sup>3</sup>Comparison between groups utilized  $\chi^2$  test;  $P > 0.05$  was considered not statistically significant.  $P < 0.05$  was considered statistically significant. COX-2: Cyclooxygenase-2.

genase (GAPDH); a negative control was established. The PCR reaction mixture of 50  $\mu$ L contained 3  $\mu$ L MgCl<sub>2</sub>, 4  $\mu$ L 10  $\times$  LA PCR buffer II (Mg<sup>2+</sup> + free), 31.75  $\mu$ L sterilized distilled water, 0.25  $\mu$ L TaKaRa LA Taq and 1  $\mu$ L the COX-2 or GAPDH primers. The PCR cycle consisted of the following steps: denaturing at 94  $^{\circ}$ C for 30 s, annealing at 60  $^{\circ}$ C for 30 s and elongation at 72  $^{\circ}$ C for 1.5 min, which was repeated for 30 cycles. Amplification products were utilized for electrophoresis in a 1.5% agarose gel, and were observed and photographed under ultraviolet light.

### Statistical analysis

Statistical analysis was performed with SPSS 13.0 software, and qualitative data was described by frequency and rate; the comparison between groups of qualitative data was made using the  $\chi^2$  test with Yates' continuity correction and Fisher's exact probability test;  $P < 0.05$  was considered significant.

## RESULTS

### mRNA expression of COX-2 in benign and malignant ascites

The positive rate of COX-2 mRNA in malignant ascites was 42.9% (9/21), which was significantly higher than in benign ascites, 6.7% (1/15), the difference being significant between the two groups ( $\chi^2 = 4.051$ ,  $P = 0.044$ ), (Table 2).

### mRNA expression of COX-2 among different disease types in benign group

Among the benign ascites, COX-2 mRNA levels were different between the tuberculous ascites (0/5) and cirrhotic ascites (1/10), but the difference being not significant ( $P = 1.000$ ), (Table 2).

### mRNA expression of COX-2 among different disease types in malignant group

The proportion of the positive rate in the malignant ascites was as follows: ovarian cancers 57.1% (4/7), colon cancer 40.0% (2/5), liver cancer 33.3% (2/6), gastric cancer 50.0% (1/2), and bladder cancer 0.00% (0/1). However, there was no significant difference in COX-2 mRNA expression among various tumors with malignant ascites ( $\chi^2 = 1.614$ ,  $P = 0.806$ ;  $P > 0.05$ ), (Table 2).

## DISCUSSION

COX, or prostaglandin-endoperoxide synthase (PGH), is a major rate-limiting enzyme in the synthesis of prostaglandin, which is able to metabolize arachidonic acid into prostaglandin products<sup>[7-9]</sup>. COX-2, an inducible protein expression, is absent in normal cells and tissues, but is rapidly synthesized and expressed under pathological conditions or after stimulation (such as inflammation, hypoxia, laser radiation, ultraviolet radiation, *etc.*). COX-2 is involved in a variety of pathophysiological processes, such as the occurrence and development of inflammation and cancer<sup>[10]</sup>. At present, mRNA expression of COX-2 in various tumor tissues has been extensively investigated; increasing numbers of research have demonstrated that COX-2 expression is unregulated in precancerous lesions and preinvasive carcinoma and positively correlates with tumor invasion and lymphatic metastasis<sup>[11-14]</sup>. Therefore, increased expression of COX-2 might occur in the early stage of the tumor and the detection of COX-2 level is helpful for early diagnosis<sup>[15,16]</sup>.

Ascites is a common symptom of many diseases and the differential diagnosis of benign and malignant ascites is important in clinical practice. So far, smear tests of exfoliated cells from ascites and the detection of tumor markers, such as CA-125, CA19-9 and AFP, have been employed to identify ascites induced by malignant tumors, but these indices are far from satisfactory in terms of sensitivity and specificity, so it is important to search for a new indicator of benign and malignant ascites<sup>[17-21]</sup>. Since COX-2 has a close relationship with tumors, its expression in malignant ascites has become an issue that is worth exploring.

We used RT-PCR to assess the mRNA expression of COX-2 in 21 cases of malignant ascites. The positive rate of COX-2 mRNA was 42.9% (9/21), which was significantly higher than in benign ascites, 6.7% (1/15) ( $P < 0.05$ ). This result indicated that the measurement of COX-2 mRNA facilitates the identification of benign and malignant ascites and has a potential value for clinical diagnosis and screening of tumors. In previous studies on COX-2, its expression was usually detected in malignant tumor tissues<sup>[22-24]</sup>, but our experiment used ascites as the samples. They were convenient to collect from patients, with less pain and being easy for clinical application. In addition, COX-2 is absent in normal cells and tissues as an inducible expression protein with specificity, so is a

potential indicator for the identification of benign and malignant ascites, and an effective supplement to common indices, such as CA125, CA19-9 and AFP.

There were no significant differences in the expression of COX-2 mRNA among various disease types with benign or malignant ascites ( $P > 0.05$ ), which was probably associated with the small number of samples and requires further confirmation. We employed one step RT-PCR, which was easy to perform, required little contact with experimental samples and avoided unnecessary contamination, and also facilitated further research and the development of clinical detection kits.

In conclusion, differential diagnosis between benign and malignant ascites is of importance and is helpful for designing a treatment plan. We hope our study can provide a new insight to explore this field in the future.

## COMMENTS

### Background

In recent years, cyclooxygenase-2 (COX-2) has been extensively studied as an inducible expression protein, and has been detected in various tumor tissues in epidemiological and cytological research. Therefore, increased expression of COX-2 might occur in the early stage of the tumor and the detection of COX-2 level is helpful for early diagnosis.

### Research frontiers

At present, mRNA expression of COX-2 in various tumor tissues has been extensively investigated; more and more research has demonstrated that COX-2 expression is upregulated in precancerous lesions and preinvasive carcinoma and positively correlates with tumor invasion and lymphatic metastasis. Therefore, increased expression of COX-2 might occur in the early stages of the tumor and the detection of COX-2 level is helpful for early diagnosis.

### Innovations and breakthroughs

This study employed RT-PCR to assess the mRNA expression of COX-2 in 21 cases of malignant ascites. The positive rate of COX-2 mRNA was 42.9% (9/21), which was significantly higher than in benign ascites, 6.7% (1/15), ( $P < 0.05$ ). This result indicated that the measurement of COX-2 mRNA facilitates the differential diagnosis between benign and malignant ascites and has a potential value for clinical diagnosis and screening of tumors.

### Applications

Differential diagnosis between benign and malignant ascites is of importance and is helpful for designing a treatment plan. The study can provide a new insight to this field in the future.

### Peer review

This is an interesting manuscript about mRNA expression of COX-2 in benign and malignant ascites. The authors made a good research on this topic. Differences in COX-2 mRNA expression among different diseases were explored. The data is well present and discussed.

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## Relationship between interleukin-6 polymorphism and susceptibility to chronic hepatitis B virus infection

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### Abstract

**AIM:** To identify the relationship between tag single nucleotide polymorphisms (tag SNPs) of interleukin-6 (*IL-6*) gene and susceptibility to chronic hepatitis B virus (HBV) infection in a Han Chinese population.

**METHODS:** We performed a case-control study of 501 Chinese patients with chronic HBV infection and 301 self-limiting HBV-infected individuals as controls. Genomic DNA was isolated from the whole blood of all subjects using phenol/chloroform with MaXtract high-density tubes. Tag SNPs were identified using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. Four tag SNPs in *IL-6* (*rs17147230A/T*, *rs2066992G/T*, *rs2069837A/G* and *rs2069852A/G*) were genotyped by the Multiplex Snap-

shot technique. The genotype and allele frequencies were calculated and analyzed.

**RESULTS:** Five haplotypes were involved in the analysis, with frequencies higher than 0.03. One of the haplotypes, TTAA, was significantly different between the two groups. Overall haplotype *P* values were: ATAA, *P* = 0.605, OR (95%CI) = 1.056 (0.860-1.297); TGAG, *P* = 0.385, OR (95%CI) = 1.179 (0.813-1.709); TGGG, *P* = 0.549, OR (95%CI) = 1.087 (0.827-1.429); TTAA, *P* = 0.004, OR (95%CI) = 0.655 (0.491-0.873); TTAG, *P* = 0.266, OR (95%CI) = 1.272 (0.832-1.944). However, the four SNPs showed no significant genotype/allele associations with susceptibility to chronic HBV infection. Overall allele *P* values were: *rs17147230*, *P* = 0.696, OR (95%CI) = 1.041 (0.850-1.276); *rs2066992*, *P* = 0.460, OR (95%CI) = 1.090 (0.868-1.369); *rs2069837*, *P* = 0.898, OR (95%CI) = 0.983 (0.759-1.274); *rs2069852*, *P* = 0.165, OR (95%CI) = 0.859 (0.693-1.064). Overall genotype *P* values were: *rs17147230*, *P* = 0.625; *rs2066992*, *P* = 0.500; *rs2069837*, *P* = 0.853; and *rs2069852*, *P* = 0.380.

**CONCLUSION:** The four tag SNPs of *IL-6* gene may be associated with susceptibility to chronic HBV infection in the Han Chinese population.

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**Key words:** Chronic hepatitis B virus infection; Interleukin-6; Single nucleotide polymorphism; Genetic susceptibility; Haplotype

**Core tip:** This study included a large number of subjects with a single ethnic background (Chinese). This would further add to the statistical power of the analysis and identify more single nucleotide polymorphisms. We selected self-limiting hepatitis B virus-infected subjects, but not unexposed subjects as controls, therefore, our results may be more reliable than other studies that

recruited blood donors as controls. In addition, we included only antiviral-naive subjects.

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## INTRODUCTION

The outcome of hepatitis B virus (HBV) infection is mainly influenced by the virus, immune response and genetic diversity<sup>[1-3]</sup>. Many studies strongly support that host genetic variety plays an important role in determining the outcome of HBV infection<sup>[4,5]</sup>. Host genetics such as single nucleotide polymorphisms (SNPs) of a variety of genes have been implicated in the diversity of HBV clinical course<sup>[6-9]</sup>. Recent studies have shown that cytokine genetic polymorphisms are associated with the development of chronic HBV infection and progression of the infection<sup>[10]</sup>.

Interleukin-6 (IL-6) is a pleiotropic cytokine with a pivotal function in regulation of the biological responses of several target cells including hepatocytes<sup>[11]</sup>. Kuo *et al.*<sup>[12]</sup> found that IL-6 was able to effectively suppress HBV replication and prevent the accumulation of HBV covalently closed circular DNA (cccDNA) in a human hepatoma cell line. Cussigh *et al.*<sup>[13]</sup> found that *IL-6* promoter polymorphisms influence the development of chronic hepatitis C virus infection. Another study suggested that *IL-6* gene polymorphisms may be associated with the outcome of allogeneic hematopoietic stem cell transplantation, particularly in patients transplanted from a related donor<sup>[14]</sup>. However, the exact mechanism involving *IL-6* in the outcome of HBV infection is unknown. The present study investigated the association between the tag SNPs of the *IL-6* gene and genetic susceptibility to chronic HBV infection.

## MATERIALS AND METHODS

### Patients

Eight hundred and two Han Chinese with HBV infection were enrolled in this study. Following recruitment, the subjects gave informed consent for genetic analysis. No abnormalities were observed in these subjects based on physical examination, chest radiography, electrocardiogram, urinalysis and routine laboratory blood testing. Liver, renal, endocrine and cardiovascular disorders were excluded. Five hundred and one were chronic HBV infected patients (221 males and 280 females). The remaining 301 were HBV natural clearance individuals and served as the control group (143 females and 158 males). The average age was 44.2 years for HBV chronic carri-

ers and 44.9 years for controls. All patients with chronic HBV infection fulfilled the diagnostic criteria: positive for hepatitis B surface antigen (HBsAg) for a period of at least 6 mo, serum HBV DNA level > 1000 copies/mL, and elevated alanine aminotransferase or aspartate aminotransferase (> 40 IU/mL). The clinical criteria for self-limiting HBV infected patients were: positive for hepatitis B surface antibody and hepatitis B core antibody, but negative for HBsAg, and no history of HBV vaccination. Controls were age- and sex-matched subjects ( $P > 0.1$ ). All cases and controls were followed for more than 6 mo. None of the patients had received anti-HBV therapy.

### Isolation of DNA from whole blood

Genomic DNA was isolated from the whole blood of all subjects using phenol/chloroform with MaXtract high-density tubes. Genomic DNA was extracted from the peripheral blood leukocyte pellet using a DNA extraction kit (Yuan Ping-Hao Biotechnology Co., Ltd. Tianjin, China), according to the manufacturer's instructions. The DNA samples were stored at -80 °C at a concentration of 100 ng/ $\mu$ L.

### Tag SNP selection

We selected SNPs on the basis of the following principal criteria: tag SNPs were identified using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The criteria for tag SNPs were  $r^2 > 0.8$ , minor allele frequency (MAF) > 0.1, functional relevance and importance, and SNPs significantly associated with diseases in previous studies. Four tag SNPs in the *IL-6* gene (*rs17147230A/T*, *rs2066992G/T*, *rs2069837A/G* and *rs2069852A/G*) were selected, which captured 100% of common SNPs (MAF > 0.1) in the HapMap Chinese database at  $r^2 > 0.8$ .

### Genotyping

The four SNPs in *IL-6* were genotyped using the Multiplex SNaPshot technique. The primers and probes were designed by Primer 5.0 software and were *rs17147230* (5' to 3'): forward primer: AAAAGGGCAAGGAAGGGAGGTA, reverse primer: CACGA GTCATTTG AGCCATCTTTG, and extension primer: TTTTTTTTTTTTTTTTTTTTTTTTGAGTT CAGTGTCATCAGCAGAACT; *rs2066992* (5' to 3'), forward primer: CTTCTGCTGGAACATTC-TATGGC, reverse primer: TTTCTGCCAGTGC CTCTTTGC, and extension primer: TTTTTTTTTTT TTTTTTTTTTTTTTTTTT TCCTAAAACAAACAC-CACTAGAGGG. *rs2069837* (5' to 3'), forward primer: CTTCTGCT GGAACATTCTATGGC, reverse primer: TTTCTGCCAGTGC CTCITTTGC, and extension primer: TTTTTTTTAAATTTGTTTTGAAGATTAG ACACAATATTTAT. *rs2069852* (5' to 3'): forward primer: CGTCATTTAACCCAGCACTTG, reverse primer: GGATTTTCTACATCAT CCCTCAGTTCC, and extension primer: TTTTTT TTTTGCCTTG-CACA CTCTTTCTG. The polymerase chain reaction

(PCR) amplification conditions were: a 15- $\mu$ L final volume containing 10  $\times$  1.5  $\mu$ L buffer, 0.3  $\mu$ L dNTPs (10 mmol/L), 0.9  $\mu$ L MgCl<sub>2</sub> (25 mmol/L), 0.1  $\mu$ L Taq DNA polymerase (TAKARA Biotechnology Co. Ltd., Dalian, China), 0.5  $\mu$ L each primer (10 pmol/L), and 1  $\mu$ L DNA template (20 mg/L). Conditions for the multiplex PCR reaction using Touch-down PCR response procedures included initial denaturation at 95 °C for 15 min, denaturation at 94 °C for 40 s, annealing at 63 °C for 1 min, and recursive-descent at 0.5 °C, followed by extension at 72 °C for 1.5 min, for a total of 15 cycles. This was followed by 25 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 8 min. Amplified samples were stored at 4 °C. After amplification, 1.5  $\mu$ L PCR product was examined on an agarose gel to test for successful amplification.

### SNaPshot reaction

The purified PCR product, each concentration of 0.2  $\mu$ mol/L SNaPshot primer mixtures, and SNaPshot fluorescent mixtures (containing Taq DNA polymerase and different fluorescently labeled ddNTP, TAKARA Biotechnology Co. Ltd., Dalian, China) consisted of a PCR system. SNaPshot response procedures were: initial denaturation at 96 °C for 10 s; denaturation at 96 °C for 10 s, annealing at 53 °C for 5 s, extension at 60 °C for 30 s, for a total of 25 cycles, and finally extension at 60 °C for 30 s. Amplified samples were stored at 4 °C. SNaPshot PCR products using SAP purification, in 10  $\mu$ L SNaPshot PCR product with 1 U SAP or 1 U CIP, were mixed and incubated at 37 °C for 1 h, and 75 °C for 15 min to inactivate the enzyme. The samples can be stored at 4 °C for 24 h or -20 °C permanently.

### DNA sequencing

The SNaPshot product was diluted 20-fold. In a total volume of 10  $\mu$ L, we mixed 8.6  $\mu$ L HiDiFormamide (high-purity formamide), 0.9  $\mu$ L GeneScan-120 LIZ Size Standard, and 0.5  $\mu$ L SNaPshot purification product. Samples were incubated at 95 °C for 5 min, chilled quickly for 4 min, and then loaded on an ABI 3730XL Genetic Analyzer (Applied Biosystems, CA, United States) for capillary electrophoresis, running GeneMapper 4.0 software for analysis of the experimental results.

### Statistical analysis

Allele and genotype frequencies were obtained by direct counting, and the  $\chi^2$  test was used to compare allele and genotype distributions. The quality of the genotype data was assessed by Hardy-Weinberg equilibrium in the case and control samples using Fisher's exact test ( $P > 0.05$ ). OR and 95%CI were calculated according to Woolf's method.

## RESULTS

We investigated the distribution of the four SNPs in

501 Chinese HBV-infected patients (cases) and 301 self-limiting HBV-infected patients (controls). All genotypes of the *IL-6* polymorphisms were in Hardy-Weinberg equilibrium in both the cases and controls.

The genotype frequencies and allele distributions of the *IL-6* polymorphisms in each subgroup of HBV-infected patients are summarized in Table 1. The genotype frequencies for AA, AT, and TT of *IL-6* rs17147230 were 30.9%, 48.1%, and 21.0% in case samples, and 28.2%, 51.5%, and 20.3% in control samples, respectively, without significant differences between cases and controls ( $P = 0.625$ ). The genotype frequencies for GG, GT and TT of *IL-6* rs2066992 were 9.0%, 37.9%, and 53.1% in case samples, and 6.6%, 39.2%, and 54.2% in control samples, respectively, without significant differences between cases and controls ( $P = 0.510$ ). The genotype frequencies for AA, AG, and GG of *IL-6* rs2069837 were 66.1%, 30.1%, and 3.8% in case samples, and 67.1%, 28.6%, and 4.3% in control samples, and no significant differences were noted ( $P = 0.853$ ). The genotype frequencies for AA, AG and GG of *IL-6* rs2069852 were 41.5%, 44.7%, and 12.8% in case samples, and 45.8%, 43.9%, and 10.3% in control samples, and no significant differences were noted ( $P = 0.380$ ). In addition, no statistically significant differences were found when the allele frequencies of SNPs rs17147230, rs2066992, rs2069837 and rs2069852 were compared between patients with chronic HBV infection and controls. Overall allele  $P$  values were: rs17147230,  $P = 0.696$ , OR (95%CI) = 1.041 (0.850-1.276); rs2066992,  $P = 0.460$ , OR (95%CI) = 1.090 (0.868-1.369); rs2069837,  $P = 0.898$ , OR (95%CI) = 0.983 (0.759-1.274); rs2069852,  $P = 0.165$ , OR (95%CI) = 0.859 (0.693-1.064).

### Haplotype analysis

We also estimated the *IL-6* haplotype frequencies and evaluated the association among these variants and HBV infection. We observed five haplotype combinations, but found no significant association in the distribution of the haplotype frequencies between cases and controls ( $P > 0.05$ ) except "TTAA" where the protective haplotype was associated with lower disease susceptibility. The haplotype "TTAA" was observed to be significantly associated with control subjects compared with patients [ $P < 0.05$ , OR (95%CI) 0.655 (0.491-0.837)]. Haplotype frequencies lower than 0.03 were ignored in the analysis (Table 2).

## DISCUSSION

Several cytokine polymorphisms are associated with the natural history of HBV infection. Zhang *et al.*<sup>[15]</sup> proved that persistent HBV infection susceptibility is associated with the gene polymorphism *IL-10* -1082 GA in the Chinese population and that clearance of HBV is associated with the gene polymorphism *IL-10* -592 CA in the Chinese population. *IL-10* -1082 G/G and *IL-12 $\beta$*  -10993 C/G are associated with early, spontaneous HBeAg seroconversion<sup>[16]</sup>. Another study indicated that the -148C,

**Table 1** Genotype and allele distributions of interleukin-6 tag single nucleotide polymorphisms in patients with chronic hepatitis B virus infection and those with self-limiting hepatitis B virus infection *n* (%)

IL-6 SNP site	Chronic HBV infection	Self-limiting HBV infection	<i>P</i> value	OR (95%CI)
rs17147230	<i>n</i> = 501	<i>n</i> = 301		
AA	155 (0.309)	85 (0.282)		1.0
AT	241 (0.481)	155 (0.515)	0.348	0.853 (0.611-1.189)
TT	105 (0.210)	61 (0.203)	0.784	0.944 (0.625-1.425)
A	551 (0.550)	325 (0.540)	0.696	1.041 (0.850-1.276)
T	451 (0.450)	277 (0.460)		
rs2066992				
GG	45 (0.090)	20 (0.066)		1.0
GT	190 (0.379)	118 (0.392)	0.252	0.716 (0.403-1.271)
TT	266 (0.531)	163 (0.542)	0.261	0.725 (0.414-1.272)
G	280 (0.279)	158 (0.262)	0.460	1.090 (0.868-1.369)
T	722 (0.721)	444 (0.738)		
rs2069837				
AA	331 (0.661)	202 (0.671)		1.0
AG	151 (0.301)	86 (0.286)	0.670	1.072 (0.780-1.472)
GG	19 (0.038)	13 (0.043)	0.758	0.892 (0.431-1.845)
A	813 (0.811)	490 (0.814)	0.898	0.983 (0.759-1.274)
G	189 (0.189)	112 (0.186)		
rs2069852				
AA	208 (0.415)	138 (0.458)		1.0
AG	229 (0.457)	132 (0.439)	0.364	1.151 (0.850-1.559)
GG	64 (0.128)	31 (0.103)	0.198	1.370 (0.848-2.213)
A	645 (0.644)	408 (0.678)	0.165	0.859 (0.693-1.064)
G	357 (0.356)	194 (0.322)		

IL-6: Interleukin-6; HBV: Hepatitis B virus; SNP: Single nucleotide polymorphism.

**Table 2** Distribution of haplotypes of interleukin-6 tag single nucleotide polymorphisms in patients with chronic hepatitis B virus infection and those with self-limiting hepatitis B virus infection

Haplotypes	Frequency (cases)	Frequency (controls)	$\chi^2$	<i>P</i> value	OR (95%CI)
ATAA	526.10 (0.525)	306.81 (0.510)	0.267	0.6051	1.056 (0.860-1.297)
TGAG	89.34 (0.089)	45.98 (0.076)	0.753	0.3854	1.179 (0.813-1.709)
TGGG	172.46 (0.172)	96.25 (0.160)	0.358	0.5494	1.087 (0.827-1.429)
TTAA	117.81 (0.118)	101.19 (0.168)	8.380	0.0038	0.655 (0.491-0.873)
TTAG	70.30 (0.070)	33.8 (0.056)	1.237	0.2660	1.272 (0.832-1.944)

+8925G and +13925C alleles of the *IL-18* gene are likely associated with HBV clearance in a Korean population<sup>[17]</sup>. However, Lee *et al*<sup>[18]</sup> found that the polymorphisms near the *IL-28B* gene, rs8099917T>G, rs12979860C>T and rs12980275A>G, are not significantly associated with the natural course of chronic HBV infection.

IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine. Hösel *et al*<sup>[19]</sup> found that IL-6 ensures early control of the virus, limiting activation of the adaptive immune response and preventing death of HBV-infected hepatocytes. IL-6 may play an extremely important role in determining liver progression<sup>[20]</sup>. Polymorphism of the *IL-6* promoter -572 loci may be associated with HCC occurrence in men<sup>[21]</sup>. Fabris *et al*<sup>[22]</sup> found that fewer patients aged < 50 years who carried one of the IL-6 high producer (G/G or G/C) genotypes experienced HBsAg loss in comparison with patients aged > 50 years and/or carriers of the IL-6 low producer C/C genotype. This indicates that possessing an IL-6 low producer phenotype may provide some advantage to older patients with

chronic HBV infection. This is consistent with other findings which showed that *IL-6* -174 G>C polymorphism may play a role in the clinical evolution of HBV infection at least in European countries where a higher prevalence of the C allele was detected in comparison with patients from the Far East<sup>[23]</sup>. However, Park *et al*<sup>[24]</sup> found that variants in the *IL-6* gene are not associated with subsequent HBV outcomes, although *IL-6* has been found to be functionally significant in other diseases<sup>[25-27]</sup>.

To identify the relationship between the SNPs of *IL-6* gene and genetic susceptibility to chronic hepatitis B virus infection in the Han Chinese population, we selected four SNPs in *IL-6* (rs1714230, rs2066992, rs2069837 and rs2069852) using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The four tag SNPs captured 100% of common SNPs (minor allele frequency > 0.1) in the HapMap Chinese database at  $r^2 > 0.8$ . We analyzed the associations of four SNP alleles with chronic HBV infection compared with self-limiting HBV infection. The results indicated that tag

SNPs of *IL-6* may be a prognostic factor for chronic hepatitis B infection. There are several advantages of this study which strengthen it compared with previous similar studies, and increase the reliability of our results. First, our study included a large number of subjects with a single ethnic background (Chinese). This would further add to the statistical power of the analysis and identify more SNPs. Second, we selected self-limiting HBV-infected subjects, but not unexposed subjects, as controls, therefore, our results may be more reliable than other studies that recruited blood donors as controls. Third, we included only antiviral-naïve subjects. In contrast, previous similar studies which investigated the natural course of chronic HBV infection did not set any limitation regarding antiviral treatment.

In conclusion, *IL-6* is a functional gene which plays a relevant role in the pathogenesis of HBV. Our study demonstrates that the tag SNPs of *IL-6* may be related to genetic susceptibility to chronic HBV infection in Chinese patients. Further genetic studies are needed to examine other SNPs in *IL-6* and their possible association with disease progression in chronic HBV infection.

## COMMENTS

### Background

Persistent hepatitis B virus (HBV) infection is considered a multifactorial and polygenic disorder with viral, environmental, and genetic components, as well as contributions from HBV genomic variability, host age, gender, concurrent infection with the hepatitis C virus, hepatitis D virus, and human immune deficiency virus. Interleukin-6 (IL-6) plays an important role in the response of the innate immune system to viral infection. *IL-6* polymorphisms affect induction of IL-6 expression.

### Research frontiers

This study is the first to investigate the association between four tag single nucleotide polymorphisms (tag SNPs) (*rs17147230A/T*, *rs2066992G/T*, *rs2069837A/G* and *rs2069852A/G*) of *IL-6* and genetic susceptibility to chronic HBV infection in Chinese patients using the Multiplex SNaPshot technique.

### Innovations and breakthroughs

The four tag SNPs of *IL-6* may be related to genetic susceptibility to chronic HBV infection.

### Applications

Based on the results of this study, further genetic studies are needed to examine the roles of other *IL-6* SNPs and their association with disease progression in chronic HBV infection.

### Terminology

IL-6 is a pleiotropic cytokine with a pivotal function in regulation of the biological responses of several target cells including hepatocytes. It acts as both a pro-inflammatory and anti-inflammatory cytokine.

### Peer review

The manuscript analyzed the association between polymorphisms of *IL-6* and clinical outcomes of HBV infection. Four SNPs of *IL-6* were genotyped using Multiplex SNaPshot technique and compared between chronic HBV infection and self-limiting HBV infection patients. The overall data showed that SNPs of *IL-6* may affect the outcome of HBV infection. The data may have a significant clinical implication.

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## Abnormal DNA-PKcs and Ku 70/80 expression may promote malignant pathological processes in gastric carcinoma

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### Abstract

**AIM:** To determine the expression of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and the Ku70/Ku80 heterodimer (Ku 70/80) in gastric carcinoma.

**METHODS:** Gastric biopsies were obtained from 146 gastric carcinoma patients [*Helicobacter pylori* (*H. pylori*)-negative: 89 and *H. pylori*-positive: 57] and 34 from normal subjects (*H. pylori*-negative: 16 and *H. pylori*-positive: 18) via surgery and endoscopic detection from April 2011 to August 2012 at the First Affiliated Hospital of Nanchang University. Pathological diagnosis and classification were made according to the criteria of the World Health Organization and the updated Sydney system. An "in-house" rapid urease test and modified Giemsa staining were employed to detect *H. pylori* infection. The expression of DNA-PKcs and the Ku 70/80 protein was detected by immunohistochemistry.

**RESULTS:** Overall, the positive rates of both DNA-PKcs and Ku 70/80 were significantly increased in gastric cancer ( $\chi^2 = 133.04$ ,  $P < 0.001$  for DNA-PKcs and  $\chi^2 =$

13.06,  $P < 0.01$  for Ku) compared with normal gastric mucosa. There was hardly any detectable expression of DNA-PKcs in normal gastric mucosa, and the positive rate of DNA-PKcs protein expression in patients with a normal gastric mucosa was 0% (0/34), whereas the rate in gastric cancer (GC) was 93.8% (137/146). The difference between the two groups was statistically significant. Additionally, the positive rate of Ku 70/80 was 79.4% (27/34) in normal gastric mucosa and 96.6% (141/146) in gastric cancer. The DNA-PKcs protein level was significantly increased in gastric cancer (Mann-Whitney  $U = 39.00$ ,  $P < 0.001$ ), compared with normal gastric mucosa. In addition, there was a significant difference in the expression of Ku 70/80 (Mann-Whitney  $U = 1117.00$ ,  $P < 0.001$ ) between gastric cancer and normal gastric mucosa. There was also a significant difference in Ku70/80 protein expression between GC patients with and without *H. pylori* infection ( $P < 0.05$ ). Spearman analysis showed a negative correlation between tumor differentiation and DNA-PKcs expression ( $r = -0.447$ ,  $P < 0.05$ ). Moreover, Ku70/80 expression was negatively correlated with both clinical stage ( $r = -0.189$ ,  $P < 0.05$ ) and *H. pylori* colonization ( $r = -0.168$ ,  $P < 0.05$ ).

**CONCLUSION:** Overall, this research demonstrated that enhanced DNA-PKcs and Ku 70/80 expression may be closely associated with gastric carcinoma.

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**Key words:** *Helicobacter pylori*; Catalytic subunit of the DNA-dependent protein kinase; Ku70/Ku80 heterodimer; Gastric carcinoma; Immunohistochemistry

**Core tip:** This is the first study to clarify the two key promoters of the non-homologous end joining (NHEJ) pathway, DNA-dependent protein kinase (DNA-PKcs) and Ku 70/80, in human gastric carcinoma tissues. The present study found an upregulated expression of DNA-PKcs and Ku 70/80 in gastric cancer tissues compared

with normal gastric mucosa, which suggests that there is an enhanced function of NHEJ in gastric carcinogenesis. As NHEJ is an error-prone and non-specific repair mechanism and can be induced before homologous recombination, its excessive activation is capable of regulating cell cycle arrest, cell apoptosis, chromosome recombination and genome instability.

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## INTRODUCTION

Gastric carcinoma is a worldwide malignant disease with high incidence and mortality, and it is characterized by genome instability through severe DNA damage caused by various factors, including *Helicobacter pylori* (*H. pylori*) infection, heredity and living habits<sup>[1,2]</sup>. The catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) and the Ku 70/Ku 80 heterodimer (Ku 70/80) can instantly combine into DNA-dependent protein kinase (DNA-PK), which is a crucial promoter of the non-homologous end joining (NHEJ) pathway<sup>[3,4]</sup>. First, DNA-PKcs receives a DNA damage signal and launches a damage response. Next, Ku 70/80 binds to the damaged DNA ends and then attracts DNA-PKcs to form DNA-PK, which can trigger NHEJ repair activities<sup>[4,5]</sup>. Accumulating evidence has shown that dysregulation of DNA-PKcs and Ku 70/80 is associated with pathological processes in various tumors<sup>[6]</sup>. The expression changes and biological function of DNA-PKcs and Ku 70/80 in gastric cancer (GC) are still unclear. It is possible that DNA-PKcs and Ku 70/80 play a dual role in tumorigenicity, as both a rapid repair method and error-prone mechanism. The inability to activate the promoter of this repair pathway could increase the mutation rate within the genome, promoting malignant cellular changes associated with carcinogenesis. Malignant tissues induce unbalanced NHEJ activities to handle metabolic stress and promote tumor infiltration. However, normal repair activities within tumor tissues may also lead to cell death and genome instability, as well as create a microenvironment that is predisposed to cancer. To determine a possible pathological role of DNA-PKcs and Ku 70/80-mediated DNA repair pathways in human gastric cancer tissues and to verify whether *H. pylori* infection disturbs the regular repair function of gastric mucosal epithelial cells through a series of DNA damage responses and the NHEJ repair pathway, we measured the expression of DNA-PKcs and Ku 70/80 by immunohistochemistry in biopsies or surgical specimens of 180 patients with or without gastric carcinoma.

## MATERIALS AND METHODS

### Patient tissue

Gastric samples of patients who underwent upper gastroduodenoscopy from January 2007 to September 2009 at The First Affiliated Hospital of Nanchang University were retrospectively reviewed and examined. A total of 180 patients (76 females and 104 males, with a mean age of  $55.37 \pm 14.05$  years) were enrolled in this study, including 34 patients with a normal gastric mucosa (NGM, *H. pylori*-negative: 16 cases and *H. pylori*-positive: 18 cases), and 146 with gastric carcinoma (GC, *H. pylori*-negative: 89 cases and *H. pylori*-positive: 57 cases). There was no significant difference in the age or gender distribution among these groups. None of the patients had been treated with any regimens to eradicate *H. pylori* infection. This study was approved ethically by the First Affiliated Hospital of Nanchang University. All of the patients gave written informed consent to participate in the study.

### Detection of *H. pylori* infection

An “in-house” rapid urease test (RUT) and modified Giemsa staining were employed for detecting *H. pylori* infection. The effectiveness of RUT is more than 95% (data not shown). The modified Giemsa staining was carried out in a double-blind fashion. An *H. pylori* infection was diagnosed as positive only if both of the methods produced positive results. An *H. pylori*-negative diagnosis was confirmed if both of the methods yielded negative results<sup>[7]</sup>.

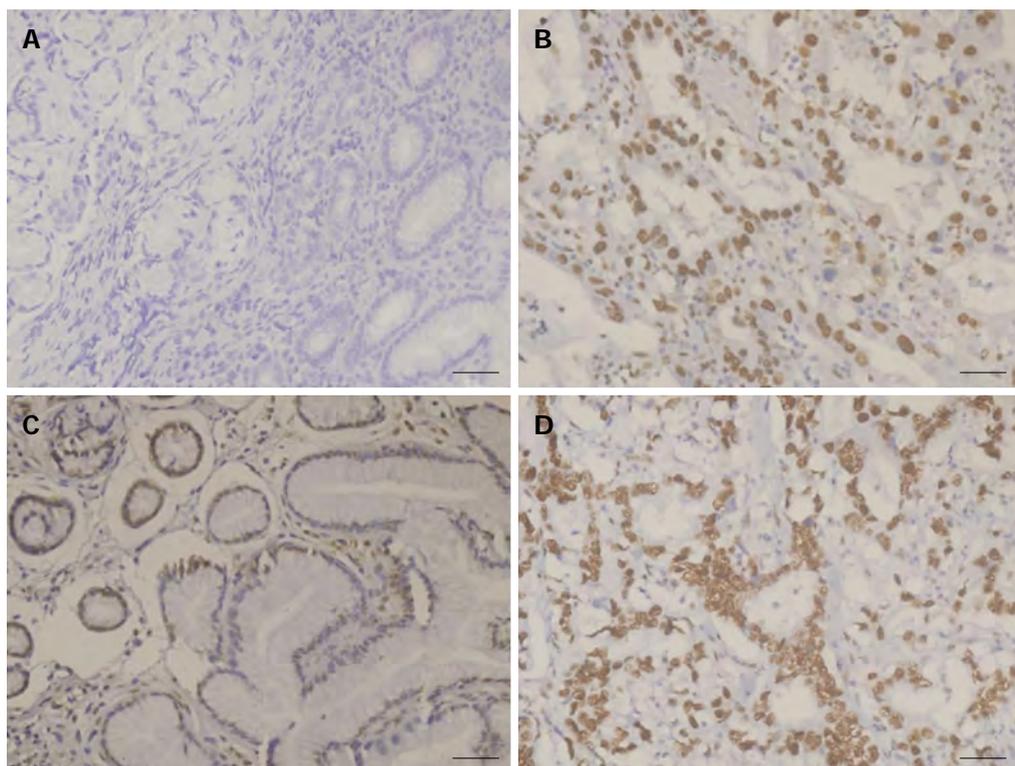
### Histological examinations of gastric samples

Gastric samples were obtained from patients who underwent endoscopy of the upper gastrointestinal tract. All of the biopsies were taken from the gastric antrum and the lesions of individual patients. The tissues used for histological analysis were fixed in 10% formaldehyde in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free phosphate-buffered saline (PBS) overnight at 4 °C before paraffin embedding. Paraffin sections of 4  $\mu\text{m}$  were cut with a microtome and stored at room temperature. Pathological diagnosis and classification were performed according to the criteria of the World Health Organization<sup>[8,9]</sup>.

### Immunohistochemistry

Primary antibodies used in this study were as follows: mouse monoclonal antihuman DNA-PKcs (Abcam, Cambridge, United Kingdom) and rabbit polyclonal antihuman Ku 70/Ku 80 (Anbobio, San Francisco, CA). The anti-human DNA-PKcs antibody was diluted 1:400, and the anti-human Ku70/80 antibody was diluted 1:3500.

The paraffin sections were mounted on slides and dewaxed in xylene and sequentially dehydrated in 100%, 95% and 85% ethanol. The sections were stained using the PV-6000 Polymer Detection System (Zhongshan Goldenbridge, Beijing, PRC) staining protocol. The sections were then washed in PBS, and endogenous peroxidase was blocked using 3%  $\text{H}_2\text{O}_2$ . After the specimens



**Figure 1** Immunohistochemistry assay of DNA-dependent protein kinase and Ku 70/80 protein expression. Immunostaining showing that the expression of DNA-dependent protein kinase was significantly upregulated in gastric cancer (B) compared to normal gastric mucosa (A). Ku 70/80 expression was significantly increased in gastric cancer (D) compared to normal gastric mucosa (C). Scale bar = 40  $\mu\text{m}$ .

were incubated with the primary antibody overnight at 4 °C, they were washed with PBS, followed by incubation with polymer helper for 30 min and poly peroxidase-antimouse or rabbit IgG for 30 min. After the sections were washed with PBS, they were incubated with 3,3-diaminobenzidine (DAB, Zhongshan Goldenbridge). The control sections incubated with PBS instead of the primary antibodies were used as negative controls. The sections were counterstained with hematoxylin.

The slides were examined under a light microscope. The cells that stained a yellow or brown color in the nucleus and/or cytoplasm were defined as positive. Five randomly selected fields per section were analyzed. In a randomly selected field from representative areas, the immunoreactive cells among 100 cells were assessed and quantified by percentage. Then, the average percentage of the five fields was used to assess the area of immunostaining (0 = 0%-5%; 1 = 6%-25%; 2 = 26%-50%; 3 = 51%-75%; 4 = 76%-100%). In addition, the intensity of immunostaining was also semi-quantitatively assessed (0 = negative, 1 = weak, 2 = moderate, 3 = intense). Then, the integrals of the “area  $\times$  intensity” were calculated, by which the overall expression levels of the proteins in the section were defined as follows: negative (-): score 0-2; weakly positive (+): score 3-5; moderately positive (++) : score 6-8; and strongly positive (+++) : score 9-12. The positive rate was the sum of weakly, moderately and strongly positive staining. The assessment of the sections was performed blindly by two pathologists, and when two

views were not consistent, the assessment was judged by a third person.

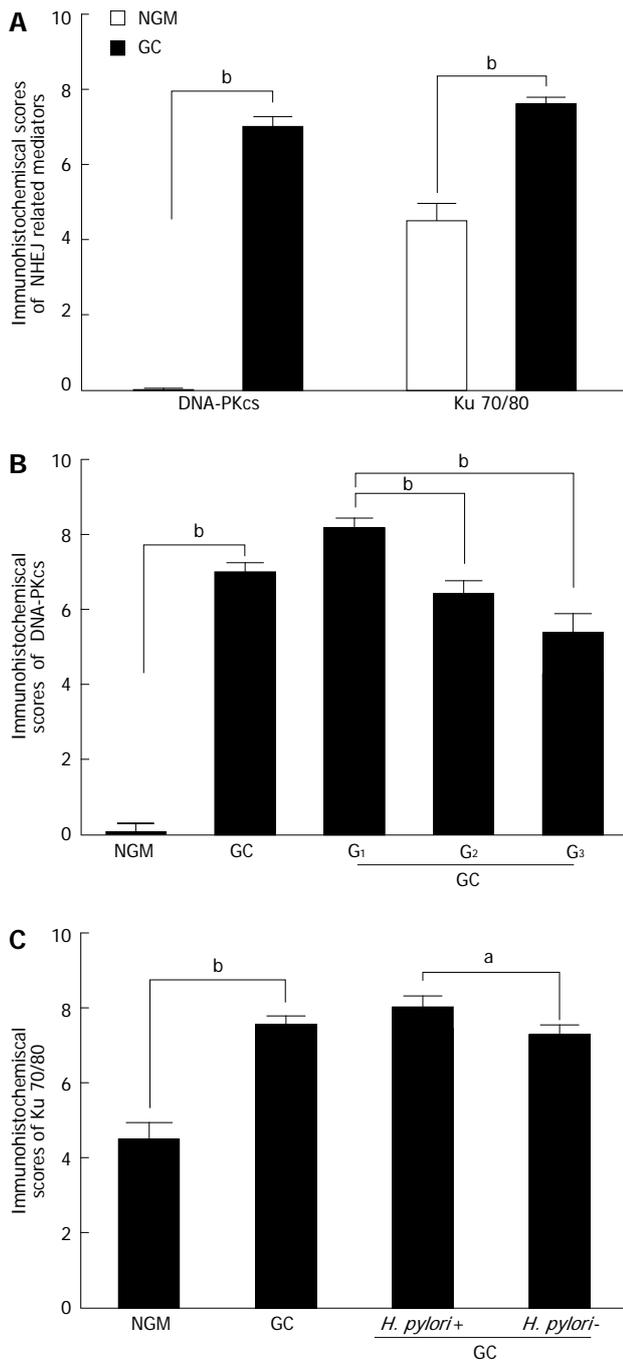
### Statistical analysis

All of the data are presented as the mean  $\pm$  SD or percentage. The  $\chi^2$  test (SPSS v.16.0 for Windows; SPSS, Inc., Chicago, IL) was used to evaluate the difference in categorical variables, such as the positive rate between groups. The Mann-Whitney *U* test (SPSS v.16.0) was used to determine the differences in numerical variables between differently defined groups. Correlations were analyzed using Spearman's rank correlation co-efficient (SPSS v.16.0). A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### Overall expression of DNA-PKcs and Ku 70/80 in gastric mucosa tissues

Overall, the positive rates of both DNA-PKcs and Ku 70/80 were significantly increased in GC ( $\chi^2 = 133.04$ ,  $P < 0.001$  for DNA-PKcs and  $\chi^2 = 13.06$ ,  $P < 0.01$  for Ku) compared with NGM. In fact, there was hardly any detectable expression of DNA-PKcs in normal gastric mucosa, and the positive rate of DNA-PKcs protein expression in patients with NGM was 0% (0/34), whereas the rate in GC was 93.8% (137/146). The difference between the two groups is statistically significant. Additionally, the positive rate of Ku 70/80 was 79.4% (27/34) in NGM



**Figure 2** Comparison of the average expression levels of DNA-dependent protein kinase and Ku 70/80 in gastric carcinoma and normal gastric mucosa tissues. **A:** Immunohistochemistry scores of DNA-dependent protein kinase (DNA-PKcs) and Ku 70/80 in 146 cases of gastric carcinoma (GC) tissues compared with matched normal gastric mucosa; **B:** Comparison of the average expression level of DNA-PKcs between different differentiation degrees from well-differentiated (G1), moderately differentiated (G2) to poorly differentiated (G3) tumor tissues; **C:** Relative expression of Ku 70/80 in *H. pylori*-positive (*H. pylori*+) and -negative (*H. pylori*-) GC tissues. The data are shown as the mean values of each group, and the error bars represent SD. Statistical analysis was performed using the Kruskal-Wallis H test and Mann-Whitney U test. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 between groups. NGM: Normal gastric mucosa.

and 96.6% (141/146) in GC (Figure 1, Table 1). The average immunohistochemistry scores of the DNA-PKcs protein were significantly increased in GC (Mann-Whitney *U* = 39.00, *P* < 0.001), compared with NGM (Figure 2A, Table 1). In addition, there was a sig-

**Table 1** Differential immunohistochemical positive rate of DNA-dependent protein kinase and Ku 70/80 in gastric carcinoma and normal gastric mucosa tissues

Group	n	DNA-PKcs				P value	Ku 70/80				P value
		-	+	++	+++		-	+	++	+++	
NGM	34	34	0	0	0	< 0.01	7	12	12	3	< 0.01
GC	146	9	24	65	48		5	19	50	72	
<i>H. pylori</i> positive											
NGM	16	16	0	0	0	< 0.01	2	4	8	2	< 0.10
GC	57	3	6	26	22		1	4	19	33	
<i>H. pylori</i> negative											
NGM	18	18	0	0	0	< 0.01	5	8	4	1	< 0.01
GC	89	6	18	39	26		4	15	31	39	

*H. pylori*: *Helicobacter pylori*; GC: Gastric carcinoma; NGM: Normal gastric mucosa; DNA-PKcs: DNA-dependent protein kinase.

**Table 2** Correlation between clinicopathological parameters and the expression of DNA-dependent protein kinase and Ku 70/80 in gastric carcinoma by Spearman analysis

Clinicopathological parameters	DNA-PKcs		Ku 70/80	
	r	P value	r	P value
Age (yr)	0.557	0.049	0.083	0.317
Sex	0.073	0.382	-0.052	0.531
<i>H. pylori</i> infection	-0.139	0.102	-0.168	0.043
Lesion location	0.131	0.115	0.107	0.200
Histological type	0.046	0.583	0.008	0.922
Local Invasive	0.042	0.615	-0.052	0.534
Differentiation degree	-0.447	< 0.001	0.068	0.414
Lymph node metastasis	0.029	0.729	0.123	0.139
Clinical stages	0.010	0.902	-0.189	0.022

*H. pylori*: *Helicobacter pylori*; DNA-PKcs: DNA-dependent protein kinase.

nificant difference in the average immunohistochemistry scores of Ku 70/80 (Mann-Whitney *U* = 1117.00, *P* < 0.001) between GC and NGM (Figure 2A, Table 1).

**Correlation between the expression of DNA-PKcs and Ku 70/80 and different clinical parameters in gastric carcinoma patients**

Overall, DNA-PKcs expression was negatively correlated with differentiation degree (*r* = -0.447, *P* < 0.01) in patients with GC (Figure 2B, Table 2). Its expression increased from well differentiated (G1) tumor tissues to moderately differentiated (G2) tissues and from G1 to poorly differentiated (G3) tumor tissues, but there was no significant increase from G2 to G3 (Table 3). Ku 70/80 expression was negatively correlated with *H. pylori* infection (*r* = -0.168, *P* = 0.043) and clinical stage (*r* = -0.189, *P* = 0.022) in patients with GC (Figure 2C, Table 2). There was no correlation between DNA-PKcs expression and Ku 70/80 expression.

**DISCUSSION**

NHEJ is a key source of genomic rearrangements, which are typically found in cancer cells<sup>[10]</sup>. Presently, numerous reports have demonstrated that chromosome transloc-

**Table 3** Expression of DNA-dependent protein kinase and Ku 70/80 in relation to the clinicopathological parameters of patients with gastric carcinoma

Clinicopathological parameters	n	DNA-PKcs				P value	Ku 70/80				P value
		-	+	++	+++		-	+	++	+++	
Age (yr)											
< 55	60	5	9	28	18	0.555	2	10	21	27	0.315
≥ 55	86	4	15	37	30		3	9	29	45	
Sex						0.380					0.530
Male	96	5	20	40	31		3	10	35	48	
Female	50	4	4	25	17		2	9	15	24	
<i>H. pylori</i> infection						0.116					0.044
Positive	57	3	6	26	22		1	4	19	33	
Negative	89	6	18	39	26		4	15	31	39	
Lesion location						0.114					0.199
Antrum	74	3	16	36	19		3	8	32	31	
Others	72	6	8	29	29		2	11	18	41	
Histological type											
Protrude <sup>1</sup>	15	3	1	6	5	0.840 <sup>1-2</sup>	0	4	4	7	0.623 <sup>1-2</sup>
Ulcerative <sup>2</sup>	57	2	10	29	16	0.586 <sup>1-3</sup>					0.546 <sup>1-3</sup>
Infiltrative ulcer <sup>3</sup>	59	4	9	24	22	0.792 <sup>1-4</sup>					0.911 <sup>1-4</sup>
Diffuse infiltration <sup>4</sup>	15	0	4	6	5	0.946 <sup>2-3</sup>	2	6	21	28	0.894 <sup>2-3</sup>
						0.946 <sup>2-4</sup>	1	6	23	29	0.655 <sup>2-4</sup>
						0.791 <sup>3-4</sup>	2	3	2	8	0.591 <sup>3-4</sup>
Local invasion											
Mucosa and submucosa <sup>1</sup>	14	2	2	6	4	0.642 <sup>1-2</sup>	0	1	5	8	0.501 <sup>1-2</sup>
Muscular layer <sup>2</sup>	21	1	4	9	7	0.518 <sup>1-3</sup>	1	2	8	10	0.395 <sup>1-3</sup>
Serosa and subserosa <sup>3</sup>	111	6	18	50	37	0.926 <sup>2-3</sup>	4	16	37	54	0.954 <sup>2-3</sup>
Differentiation degree											
G <sub>1</sub> <sup>1</sup>	64	3	3	22	36	0.000 <sup>1-2</sup>	1	6	24	33	0.321 <sup>1-2</sup>
G <sub>2</sub> <sup>2</sup>	57	4	13	29	11	0.000 <sup>1-3</sup>	3	6	23	25	0.618 <sup>1-3</sup>
G <sub>3</sub> <sup>3</sup>	25	2	8	14	1	0.146 <sup>2-3</sup>	1	7	3	14	0.866 <sup>2-3</sup>
Lymph node metastasis						0.728					0.318
Positive	113	6			36		5	15	41	52	
Negative	33	3			12		0	4	9	20	
Clinical stages											
I <sup>1</sup>	13	2			4	0.539 <sup>1-2</sup>	0	1	5	7	0.673 <sup>1-2</sup>
II <sup>2</sup>	46	2			14	0.545 <sup>1-3</sup>	2	3	12	29	0.382 <sup>1-3</sup>
III <sup>3</sup>	34	1			12	0.638 <sup>1-4</sup>	0	3	18	13	0.243 <sup>1-4</sup>
IV <sup>4</sup>	53	4			18	0.983 <sup>2-3</sup>	3	12	15	23	0.075 <sup>2-3</sup>
						0.832 <sup>2-4</sup>					0.029 <sup>2-4</sup>
						0.820 <sup>3-4</sup>					0.511 <sup>3-4</sup>

<sup>1-4</sup>Distinguish the *P* value between each histological type. DNA-PKcs: DNA-dependent protein kinase.

tions are a common cause of malignancy, and oncogenic fusion genes have been found in many hematological and solid tumors<sup>[11-13]</sup>. The over-expression of DNA-PKcs and Ku 70/80 has been found in various human tumors, but the pathways that promote translocations in gastric cancer are still unclear<sup>[14-16]</sup>. It is well-established that *H. pylori* infection is a definite etiological factor in gastric carcinogenesis<sup>[17-19]</sup>. However, the mechanism through which *H. pylori* infection contributes to the development of gastric cancer has not been fully elucidated. Obst *et al*<sup>[20]</sup> reported that *H. pylori* infection could cause DNA damage in gastric epithelial cells. Because Toller *et al*<sup>[21]</sup> showed an increased expression of  $\gamma$ -H2AX in *H. pylori*-infected GES-1 cells and indicated that GC may be associated with DNA double strand breaks (DSBs), we expected to detect increased DNA-PKcs expression in GC to explain the error-prone repair pathway found in the gastric mucosa.

In this study, we found that the expression of DNA-

PKcs and Ku 70/80 was increased in GC compared with NGM. Spearman analysis indicated a negative correlation between the expression of DNA-PKcs and tumor differentiation degrees. Moreover, Ku 70/80 was increased in *H. pylori*-positive GC patients compared with the negative group. Analysis of the data from 146 cases of GC did not show an obvious association between DNA-PKcs or Ku 70/80 expression levels and invasive lymph node metastasis or tumor histological type. We hypothesized that the enhanced DNA-PKcs and Ku 70/80 expression in gastric cancer may result from *H. pylori*-induced DNA damage in gastric epithelial cells. We initially analyzed the relationship between DNA-PKcs and Ku 70/80 protein expression and *H. pylori* infection status. As shown in the right panel of Figure 1C, the Ku 70/80 protein expression level was higher in *H. pylori*-positive GC tissues than in the *H. pylori*-negative tissue.

Numerous studies have indicated that genome instability can be induced by NHEJ, which may increase the

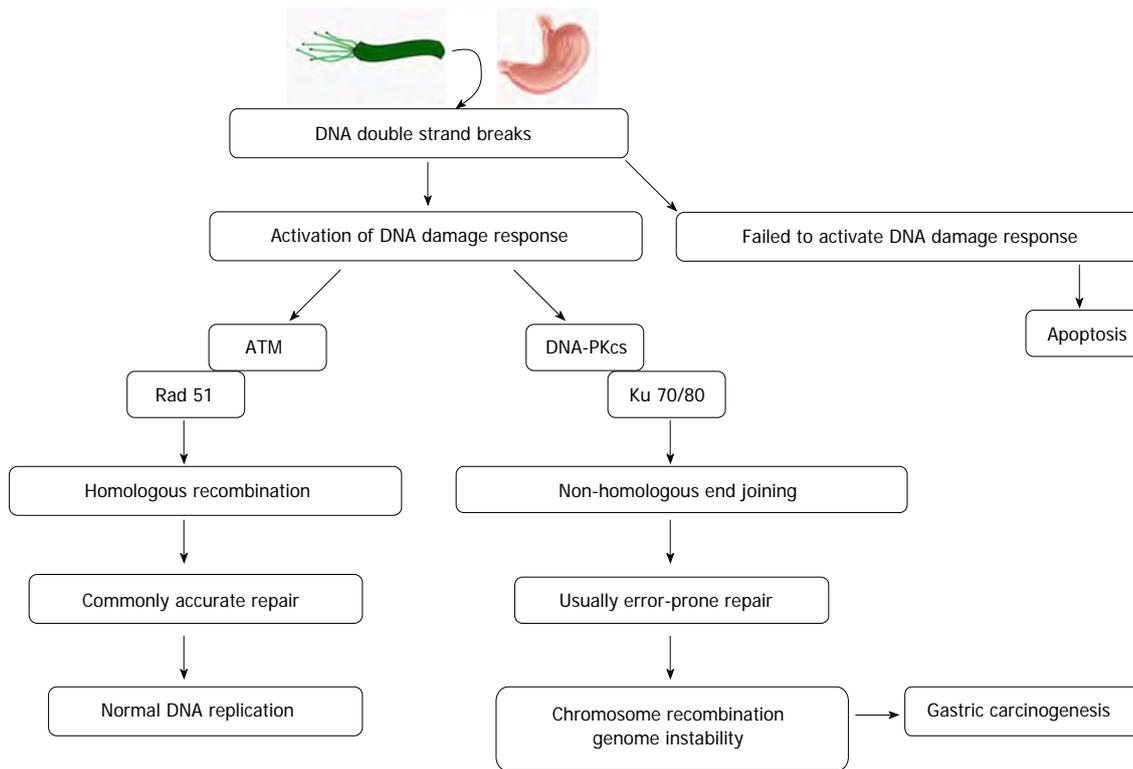


Figure 3 A model depicting the effect of *Helicobacter pylori* infection on the DNA-dependent protein kinase and Ku 70/80-mediated non-homologous end joining repair pathway in gastric epithelial cells that might ultimately promote carcinogenesis. ATM: Ataxia telangiectasia mutated; DNA-PKcs: DNA-dependent protein kinase.

risk of malignant transformation in cells<sup>[6]</sup>. In line with our results, Hu *et al.*<sup>[22]</sup> reported that the expression of Ku70 proteins in precancerous lesions and GC tissue was significantly higher than that in normal gastric mucosal tissue. Moreover, the expression of Ku70 proteins in GC tissue was significantly higher than in precancerous lesions. Lee *et al.*<sup>[23]</sup> verified that DNA-PKcs expression status was increased in consecutive cases of gastric cancer (450/564) by immunohistochemistry. Moreover, they found that DNA-PKcs expression was negative in the foveolar epithelium of normal gastric mucosal tissues but was positive in most *H. pylori*-associated gastritis, intestinal metaplasia and gastric adenoma tissues. Lim *et al.*<sup>[24]</sup> found that Ku70 and Ku80 expression is mediated by constitutively activated NF-kappaB and constitutively expressed COX-2 in gastric cancer cells and indicated that Ku70 and Ku80 expression may be related to gastric cell proliferation and carcinogenesis. These data are consistent with our findings. Here, we show that exposure to *H. pylori* significantly upregulated Ku 70/80 expression but did not influence the expression of DNA-PKcs. We speculated that a lack of effectively activated DNA-PKcs might be responsible for the severe DNA damage and abnormal NHEJ repair activities due to *H. pylori* infection in gastric epithelial cells. In addition, environmental factors, such as *H. pylori* infection, stress and behavioral habits, may upset the balance between DNA-PKcs and Ku 70/80, and thus the damaged cells cannot launch the rapid and non-specific repair mechanism, leading to more serious and irreparable damage.

To our knowledge, this is the first study to clarify the two key promoters of the NHEJ pathway in human gastric carcinoma tissue, DNA-PKcs and Ku 70/80. The upregulated expression of DNA-PKcs and Ku 70/80 in GC tissues compared with NGM confirms that there is enhanced NHEJ in human gastric carcinoma. As NHEJ is an error-prone and non-specific repair mechanism and can be induced before homologous recombination (HR), its excessive activation is capable of regulating cell cycle arrest, cell apoptosis, chromosome recombination and genome instability<sup>[25,26]</sup>. Our finding that Ku 70/80 expression is positively correlated with *H. pylori* colonization suggests that this alteration may be highly relevant for pathological processes during *H. pylori* infection. Infection with *H. pylori* can serve as a unique model system to determine the role of Ku 70/80-mediated abnormal NHEJ repair activities in gastric carcinogenesis (Figure 3).

We found the following results: (1) gastric carcinogenesis is associated with enhanced non-homologous end joining repair; and (2) *H. pylori* mainly upregulates the expression of Ku 70/80 as opposed to DNA-PKcs to trigger NHEJ in gastric epithelial cells. Our data contribute to the complexity of the NHEJ function in GC. To illustrate the molecular mechanism underlying DNA-PK function in gastric carcinogenesis, further studies with larger samples in the various stages of gastric carcinoma are needed.

In conclusion, the present study provides new perspectives into the role of DNA-PKcs and Ku 70/80 in gastric carcinogenesis. Our data indicated that dysregula-

tion of DNA-PK occurs frequently in GC and is associated with GC progression, which may be another important mechanism leading to abnormal repair activities in damaged gastric epithelial cells, especially the upregulated Ku 70/80 expression in *H. pylori*-related GC. In the current study, we found that both DNA-PKcs and Ku 70/80 were over-expressed in GC tissues. These results suggest that altered expression of DNA-PKcs and Ku 70/80 may function as a potential factor that may lead to genome instability in gastric carcinoma.

## COMMENTS

### Background

Gastric carcinoma (GC) is characterized by genome instability through severe DNA damage caused by various factors, including *Helicobacter pylori* (*H. pylori*) infection, heredity and living habits. The incidence of gastric carcinoma is currently rising rapidly throughout the world, especially in developing countries. The exact pathogenesis of this disease is still unknown. Abnormal expression of DNA-dependent protein kinase (DNA-PKcs) and Ku 70/80 is associated with tumor growth, invasion and metastasis and may also play a crucial role in error-prone DNA repair activities in gastric epithelial cells, thus leading to genome instability and gastric carcinogenesis.

### Research frontiers

DNA-PKcs and Ku 70/80 are crucial promoters of the non-homologous end joining (NHEJ) pathway. Accumulating evidence has shown that dysregulation of DNA-PKcs and Ku 70/80 is associated with pathological processes in various tumors. The expression changes and biological function of DNA-PKcs and Ku 70/80 in gastric carcinoma are still unclear. In this study, the authors demonstrate that the overexpression of DNA-PKcs and Ku 70/80 could be a potential mechanism for mediating error-prone DNA repair pathways in human gastric cancer tissues and verified that *H. pylori* infection may increase the expression of Ku 70/80, disrupting the standard activation of the NHEJ repair pathway, which is associated with gastric epithelial malignancies.

### Innovations and breakthroughs

Recent reports have highlighted the importance of genome instability-related DNA repair promoters, including DNA-PKcs and Ku 70/80, in gastric carcinogenesis. Both DNA-PKcs and Ku 70/80 are over-expressed in gastric carcinoma compared to normal gastric mucosa. This is the first study to explore the change in expression of DNA-PKcs and Ku 70/80 in gastric carcinoma, to analyze the correlation between the expression of DNA-PKcs and Ku 70/80 and to investigate a series of clinical parameters to illustrate the potential pathological mechanism of carcinogenesis.

### Applications

By verifying the changes of DNA-PKcs and Ku 70/80 and their relationship with gastric carcinoma, this study may provide new insights into the pathological mechanism of gastric cancer. Furthermore, the combination of DNA repair molecules and targeting drugs may offer a synergistic effect in the therapeutic treatment of gastric carcinoma.

### Terminology

The catalytic subunit of the DNA-PKcs and the Ku 70/Ku 80 hetero-dimer (Ku 70/80) are crucial promoters of the error-prone NHEJ pathway. First, DNA-PKcs receives a DNA damage signal and launches a damage response. Next, Ku 70/80 binds to the damaged DNA ends and then attracts DNA-PKcs to form DNA-PK, which can trigger NHEJ repair activities. Accumulating evidence has shown that dysregulation of DNA-PKcs and Ku 70/80 is associated with pathological processes in various tumors.

### Peer review

The authors examined the expression of DNA-PKcs and Ku 70/80 in gastric carcinoma. The study revealed that both DNA-PKcs and Ku 70/80 were increased in gastric carcinoma. The increase of DNA-PKcs expression was negatively correlated with differentiation degree, and Ku 70/80 expression was negatively correlated with *H. pylori* infection and the clinical stage of patients with GC. The results are interesting and may represent a new horizon for exploring the pathological mechanism of gastric carcinoma.

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## Diabetes mellitus carries a risk of gastric cancer: A meta-analysis

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### Abstract

**AIM:** To investigate the association and quantify the relationship between diabetes mellitus (DM) and gastric cancer (GC) by an updated meta-analysis.

**METHODS:** The initial PubMed search identified 1233 publications. Studies not reporting GC or those not reporting actual number of GC were excluded. Twelve pertinent studies were retrieved from the PubMed database or from a manual search and considered for the meta-analysis. Pooled risk ratios and 95%CI were estimated by a random-effects model. Subgroup analysis was performed according to gender or geographical regions. Heterogeneity and publication bias were evaluated by  $I^2$  and funnel plot analysis, respectively.

**RESULTS:** DM was significantly associated with GC with a RR of 1.41 ( $P = 0.006$ ) (95%CI: 1.10-1.81). Subgroup analyses revealed that both sexes showed a significant association with GC, with a greater magnitude of risk in females (RR = 1.90; 95%CI: 1.27-2.85;  $P = 0.002$ ) than in males (RR = 1.24; 95%CI: 1.08-1.43;  $P = 0.002$ ). In addition, the link between DM and GC was significant in East Asian DM patients (RR = 1.77; 95%CI: 1.38-2.26;  $P < 0.00001$ ) but not in Western DM patients (RR = 1.23; 95%CI: 0.90-1.68;  $P = 0.2$ ). There was no evidence of publication bias, but the re-

sults indicated significant heterogeneity.

**CONCLUSION:** This updated meta-analysis has provided evidence of positive DM-GC associations. The limited information on potentially important clinical confounding factors in each study deserves further investigation.

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**Key words:** Gastric cancer; Diabetes mellitus; Meta-analysis; Hyperglycemia; Hyperinsulinemia

**Core tip:** Diabetes mellitus (DM) was significantly associated with gastric cancer (GC) with a risk ratio of 1.41. This positive DM-GC association was also observed in both sexes with a greater magnitude of risk in females than male, and in East Asian patients but not in Western patients. This study could provide one answer to current inconsistent knowledge across trials concerning a positive/inverse DM-GC association. Since DM patients are less likely to be screened for cancers, clinicians caring for DM patients should remain alert to detect GC especially in females, since there are fewer female than male GC patients in the general population.

Shimoyama S. Diabetes mellitus carries a risk of gastric cancer: A meta-analysis. *World J Gastroenterol* 2013; 19(40): 6902-6910 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6902.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6902>

### INTRODUCTION

A growing body of evidence, derived largely from case-control studies, cohort studies, and meta-analyses, suggests that diabetes mellitus (DM) is associated with an increased risk of a number of cancers. The risk increases 2-fold for cancers of the liver, pancreas, and endome-

trium, and 1.2-1.5-fold for cancers of the colon and rectum, breast, bladder, and kidney<sup>[1]</sup>, while prostate cancer shows a positive<sup>[2]</sup> or inverse<sup>[1]</sup> association with DM. In fact, DM is a disease of global epidemic proportions. The DM population has increased from 171 million in 2000 to 366 million in 2011, a figure projected to increase to 552 million by 2030<sup>[3,4]</sup>. This increasing prevalence of DM with its anticipated 200 million more patients over the next two decades suggests that even a small increase in cancer risk will have an undeniable impact on the health of the general population. Therefore, in addition to the dramatic increase in the prevalence of DM and the consequences of its complications, a DM-cancer association may greatly affect worldwide health levels.

Despite the heightening clinical awareness of the DM-cancer association, however, the risk of gastric cancer (GC) in DM patients has seemingly attracted little attention among diabetes researchers and healthcare providers, and this topic has been scarcely addressed in the English literature with contradictory findings. This dearth of data may be attributable to the fact that the disease *per se* has been paid little attention in the West, with fewer established regular screening programs for GC. Consequently, the risk of GC in DM patients is still overshadowed by the more common acute and chronic DM complications such as cardiovascular and renal diseases, which largely account for the 2-fold increase in mortality associated with DM<sup>[5]</sup>. Under these circumstances, the reported risks of GC in DM patients have been inconsistent, being high<sup>[6,7]</sup>, neutral<sup>[8]</sup>, or inverse<sup>[9]</sup> with an odds ratio or incidence RR of 1.14 (95%CI: 1.03-1.31) to 2.07 (95%CI: 1.40-3.08), 1.2 (95%CI: 0.74-1.70) to 1.6 (95%CI: 0.79-2.32), and 0.67 (95%CI: 0.46-0.99), respectively. Mixed results were also observed while evaluating the association between fasting glucose and GC risk; the Japanese Hisayama study<sup>[10]</sup> showed a positive association, but European<sup>[11]</sup> and Korean<sup>[12]</sup> studies did not. Furthermore, the studies investigating DM-GC associations comprised heterogeneous participants without distinguishing between type 1 DM (T1DM) and type 2 DM (T2DM)<sup>[13]</sup>, or were based on different DM criteria such as treatment<sup>[14,15]</sup>, fasting blood glucose<sup>[11]</sup>, or self-report<sup>[16,17]</sup>. Even three recent meta-analyses have provided mixed results with neutral<sup>[18]</sup>, marginal<sup>[19]</sup>, and positive<sup>[20]</sup> DM-GC associations. This article aims to update the DM-GC association by including several of the most recent articles as well as others investigating the actual number of GC patients in DM and non DM cohorts.

## MATERIALS AND METHODS

All publications concerning the DM-GC association were retrieved from the English literature. A computerized literature search between the years 1950 and January 2013 was conducted in PubMed using Boolean operators, with (“cancer” or “carcinoma”) and “diabetes” as keywords. Additional studies that were considered pertinent were sought by a manual search through reference lists

in the retrieved publications. The reference retrieval was additionally complemented by a manual search of references from previous meta-analyses<sup>[18-20]</sup>. When more than one analysis of the same cohort was published, the most recent was selected. Articles which apparently reported cancers other than GC in their title/abstract were excluded. Afterwards, following a thorough review of the selected articles, 12 studies reporting comparisons on actual numbers of GC patients between DM and non DM subjects were finally judged to qualify<sup>[11,15-17,21-28]</sup>. The reference lists of the identified meta-analyses were searched to identify original research reports on this topic. Reports from Japan<sup>[16,21,22]</sup> and Taiwan<sup>[15,23]</sup> were defined as East Asian studies, and those from the United States<sup>[17,24,25]</sup> and Europe<sup>[11,26,27]</sup> defined as Western studies.

## Statistical analysis

Each GC incidence in each publication was treated as a dichotomous variable. Data from all relevant studies were combined to estimate the pooled RR with a 95%CI using the random effects model<sup>[29]</sup> provided by the Cochrane Library software Review Manager 5. An RR less than or greater than 1.0 meant respectively a negative or positive DM-GC association. Heterogeneity was quantified using the  $I^2$  measure, in which  $I^2 < 30\%$  indicated mild heterogeneity, 30%-70% moderate heterogeneity, and  $> 70\%$  severe heterogeneity<sup>[30]</sup>. Publication bias was evaluated by funnel plot analysis using Comprehensive Meta Analysis version 2 software.  $P < 0.05$  was considered significant.

## RESULTS

The initial PubMed search identified 1233 publications. After the title and abstract review, studies reporting cancers other than GC in DM patients were excluded, and 152 articles deemed potentially relevant were retrieved for further evaluation. Excluding studies not reporting the actual number of GC patients in DM and nonDM cohorts, 12 publications were ultimately selected (Figure 1), yielding a total of 16725 GC patients: 2150 DM and 14575 non-DM. T1DM and T2DM were not differentiated in these publications except for two in which only T2DM patients were investigated. Five<sup>[15,16,21-23]</sup> studies were from East Asia, 6<sup>[11,17,24-27]</sup> were from the West, and one<sup>[28]</sup> was from Israel. Each publication provided mixed results concerning the DM-GC association with adjustment of confounders (Table 1).

The pooled results showed a significant increase in GC risk in the DM cohort (RR = 1.41; 95%CI: 1.10-1.81;  $P = 0.006$ ) with significant statistical heterogeneity ( $I^2 = 95\%$ ;  $P < 0.00001$ ) (Figure 2A). The subgroup analyses stratified by gender or geographical regions revealed positive GC associations in both sexes, with a larger magnitude of correlation in females (RR = 1.90; 95%CI: 1.27-2.85;  $P = 0.002$ ) than in males (RR = 1.24; 95%CI: 1.08-1.43;  $P = 0.002$ ) (Figure 2B and C). East Asian subjects showed a 77% increased risk of GC (RR = 1.77; 95%CI: 1.38-2.26;  $P < 0.00001$ ) but Western subjects did not (RR = 1.23; 95%CI: 0.90-1.68;  $P = 0.2$ ) (Figure 2D

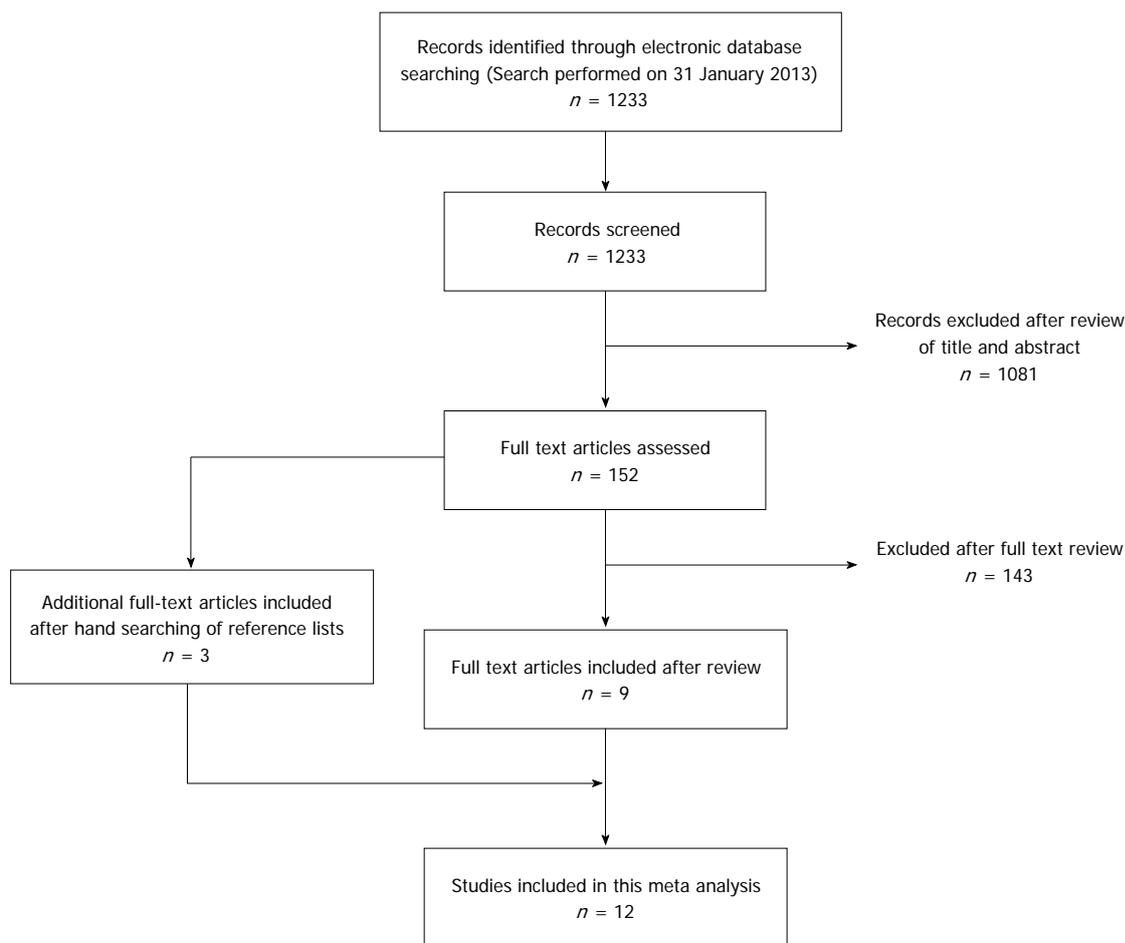


Figure 1 Flow chart of the publication selection process.

and E). The visual inspection of the funnel plots seemed basically symmetric, and Egger’s test did not indicate statistically significant asymmetry for all included studies (intercept = 0.70, one-tailed  $P = 0.37$ ), indicating no evidence of publication bias (Figure 3).

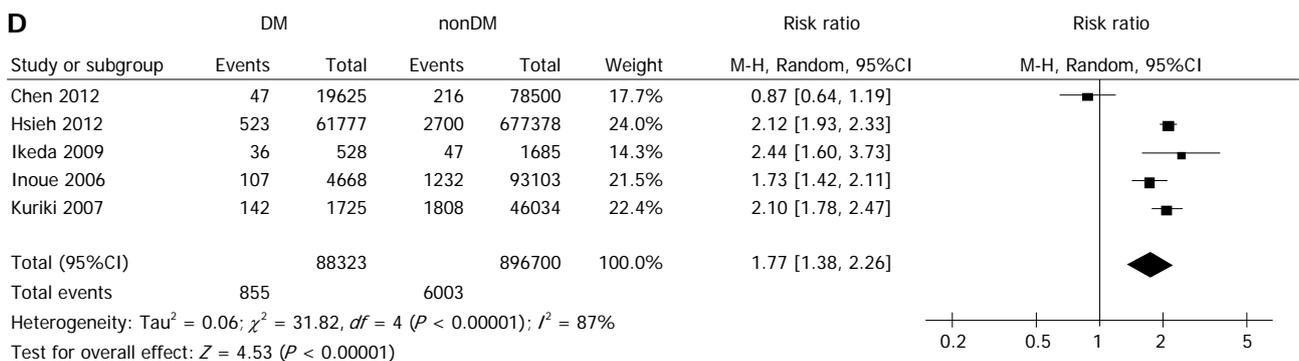
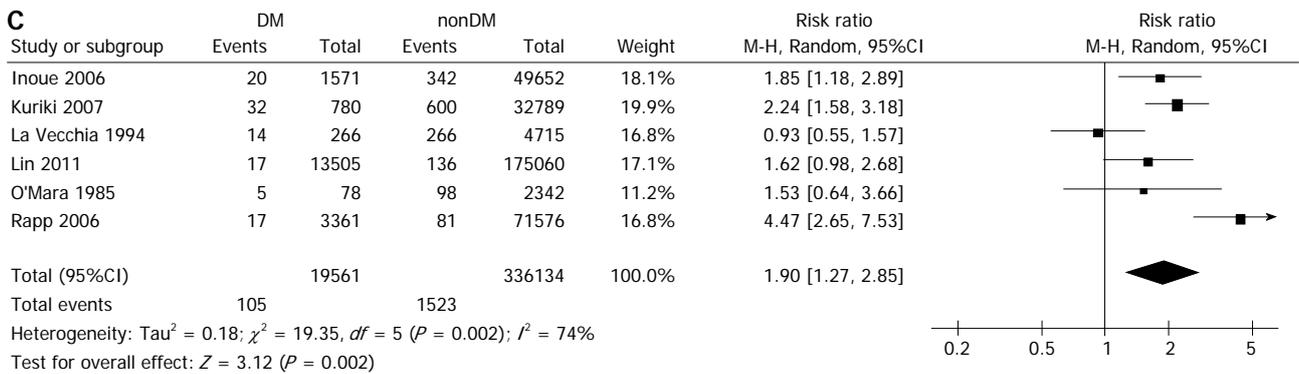
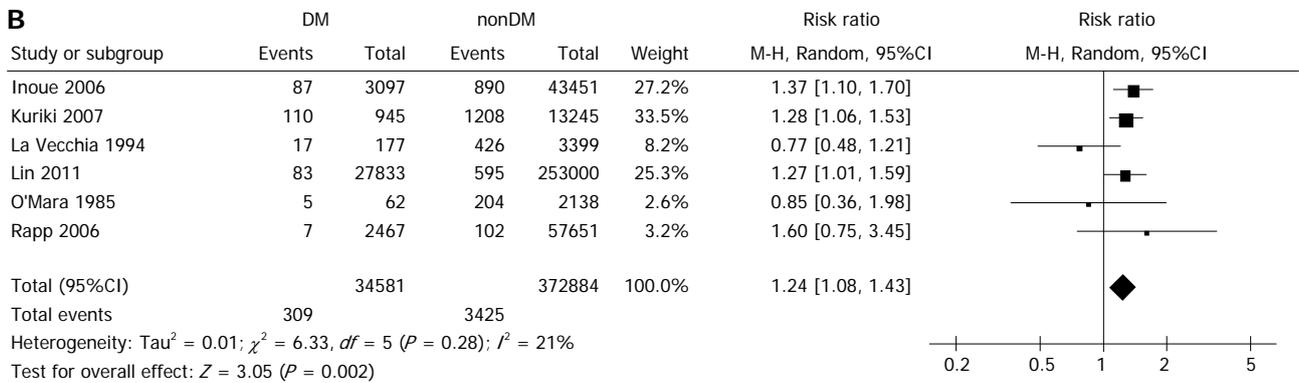
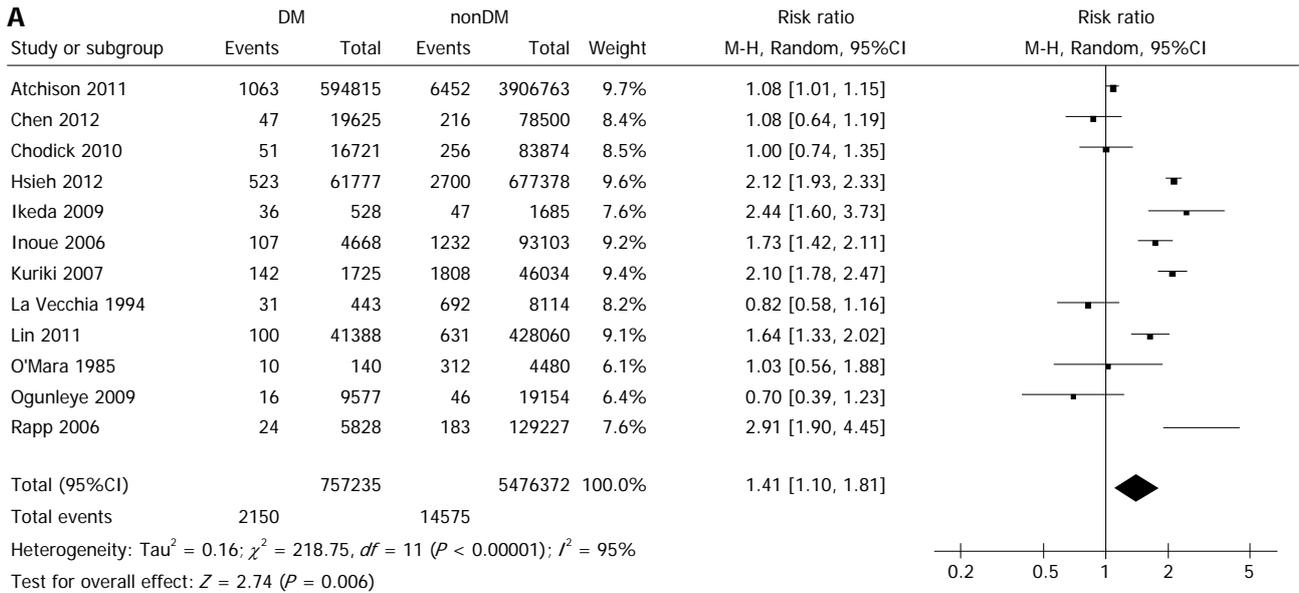
## DISCUSSION

This updated meta-analysis, with GC as the disease in focus in articles published up to January 2013, has elucidated a positive DM-GC association, the findings being consistent with one previous meta-analysis<sup>[20]</sup>. A subgroup analysis has provided the first evidence of a significantly increased risk of GC in both sexes, with a more prominent association in females than in males. Furthermore, the DM-GC association was positive for East Asians but not for Western subjects.

This meta-analysis focused on GC incidence rather than GC mortality, because GC mortality could be mainly influenced by the treatment modalities for GC such as extent of surgery and chemotherapy regimens, which differ markedly between countries. These are the reasons for the relatively fewer number of papers included in this meta-analysis compared with the previous ones<sup>[19,20]</sup>. However, against the background of controversial findings<sup>[18-20]</sup> in this matter in the literature, this study pro-

vided data supporting a positive DM-GC association.

There is a consensus that T2DM is associated with a spectrum of cancers. Although the exact underlying mechanisms linking DM and cancers remain unknown, several possible mechanisms have been debated and proposed: (1) the association between DM and cancer is direct through hyperglycemia; (2) diabetes is preceded by hyperinsulinemia and insulin resistance that alter cancer risk; and (3) the DM-cancer association is due to common risk factors such as obesity. Each of these represents a hallmark metabolic abnormality identified in T2DM and can potentially underlie the association between DM and GC. First, Swedish T1DM patients had more than twice the relative risk of GC than the general population<sup>[31,32]</sup>, suggesting that the associations between GC and hyperglycemia are biologically plausible since T1DM is an autoimmune disease manifesting as hyperglycemia due to pancreatic beta-cell destruction and insulin deficiency. Several mechanisms have been proposed that could explain the relationship between hyperglycemia and cancer. Hyperglycemia causes oxidative stress which promotes the formation of advanced glycation products (AGEs) and the expression of their receptor (RAGE); the AGE/RAGE interaction in turn stimulates oxidative stress. Furthermore, the crosstalk between the AGE/RAGE system and oxidative stress has been known to



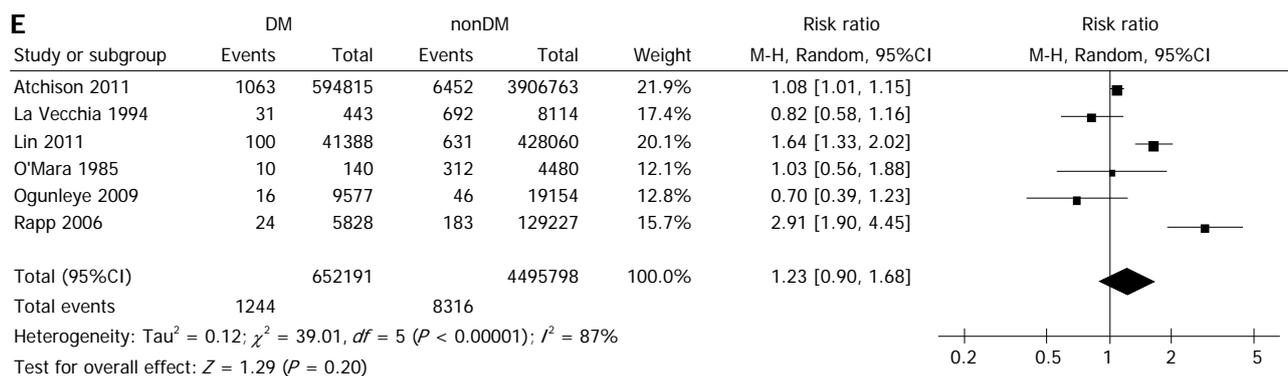


Figure 2 Forest plot representation-random-effects model. A: All included publications. Stratified by sex; B: Male; C: Female, and stratified by geographical area; D: East Asia; E: The West. The individual block squares denote the RR for each study of gastric cancer risk among diabetes mellitus (DM) patients, with an area proportional to the amount of statistical information in each study. The horizontal line denotes a 95%CI, ending with an arrowhead when CI extends beyond the scale. The pooled estimate and its 95%CI are represented by a diamond. Squares or diamonds plotted in the right half indicate increased gastric cancer risk. The risk is considered significant only if the horizontal line or diamond does not overlap the solid vertical line.

**Table 1 Summary of included studies**

Ref.	Country	Study population	Diagnosis of DM	RR of GC (95%CI)	Confounders or Adjusted factors
Atchison <i>et al</i> <sup>[24]</sup>	United States	Veteran men	Hospital disease record	0.95 (0.89-1.02)	Age, time, latency, race, number of visits, alcohol, obesity, chronic obstructive pulmonary diseases
Chen <i>et al</i> <sup>[15]</sup>	Taiwan	National Health Insurance database	Antidiabetic drug	0.90 (0.65-1.23)	Age, gastric polyp, partial gastrectomy, gastric ulcer, pneumoconiosis
Chodick <i>et al</i> <sup>[28]</sup>	Israel	Healthcare service registry	Antidiabetic drug	Men 1.44 (0.98-2.11) Women 0.99 (0.55-1.80)	Age, region, use of healthcare service, BMI, cardiovascular disease
Hsieh <i>et al</i> <sup>[23]</sup>	Taiwan	National Health Insurance database	Ambulatory or inpatient care	0.92 (0.84-1.01)	age, sex
Ikeda <i>et al</i> <sup>[21]</sup>	Japan	Hisayama, population-based	Oral glucose tolerance test, fasting plasma glucose	2.13 (1.30-3.47) <sup>1</sup> 2.69 (1.24-5.85) <sup>2</sup>	Age, sex, <i>Helicobacter pylori</i> peptic ulcer, BMI, total cholesterol, alcohol, smoking, dietary factors
Inoue <i>et al</i> <sup>[16]</sup>	Japan	Public Health Center-based prospective study	Questionnaire	Men 1.23 (0.98-1.54) Women 1.61 (1.02-2.54)	Age, study area, cerebrovascular disease, ischemic heart disease, smoking, alcohol, BMI, physical activity, green vegetable intake, coffee intake
Kuriki <i>et al</i> <sup>[22]</sup>	Japan	Hospital-based epidemiologic research program	Questionnaire	Men 1.16 (0.93-1.44) Women 1.70 (1.16-2.48)	Age, BMI, drinking and smoking, physical activity, bowel movement, family history of cancer or diabetes, dietary restriction, raw vegetable intake, greasy food intake
La Vecchia <i>et al</i> <sup>[26]</sup>	Italy	Case-control study	Questionnaire	0.6 (0.4-0.9)	age, sex
Ge <i>et al</i> <sup>[19]</sup>	United States	National Institutes of Health American Association of Retired Persons diet and health study	Questionnaire	Cardia 1.89 (1.43-2.50) Noncardia 0.98 (0.70-1.37)	Age, sex, calories, alcohol, smoking, fruit intake, vegetable intake, ethnicity, education, physical activity
O'Mara <i>et al</i> <sup>[25]</sup>	United States	Case-control study	Questionnaire	Men 0.7 (ND) Women 1.2 (ND)	Age
Ogunleye <i>et al</i> <sup>[27]</sup>	United Kingdom	Health Informatics Center	Registry	0.73 (0.41-1.29)	Deprivation decile
Rapp <i>et al</i> <sup>[11]</sup>	Austria	Vorarlberg Health Monitoring and Promotion Programme	Fasting blood glucose	Men 0.84 (0.38-1.87) <sup>3</sup> Women 1.16 (0.66-2.05) <sup>4</sup>	Age, smoking, occupational group, BMI

<sup>1</sup>Hemoglobin A1c, 6.0%-6.9%; <sup>2</sup>Hemoglobin A1c,  $\geq 7.0\%$ ; <sup>3</sup>Fasting blood glucose,  $\geq 7$  mmol/L; <sup>4</sup>Fasting blood glucose, 6.1-6.9 mmol/L. GC: Gastric cancer; ND: Not described; BMI: Body mass index; DM: Diabetes mellitus.

activate numerous cell signaling pathways related to cell growth and apoptosis<sup>[33]</sup> that could eventually promote carcinogenesis and cell invasion<sup>[34]</sup>. Indeed, *in vitro* analyses have revealed the AGE/RAGE interaction positively correlating with the invasion and metastasis of gastric<sup>[35]</sup>,

pancreatic<sup>[36]</sup>, and biliary<sup>[37]</sup> cancers. However, considering that epidemiological studies failed to find any increased risk of pancreatic, breast, colorectal, kidney, liver, or bladder cancers in T1DM patients<sup>[31,32]</sup>, which in turn are associated with cases of T2DM, and that the association

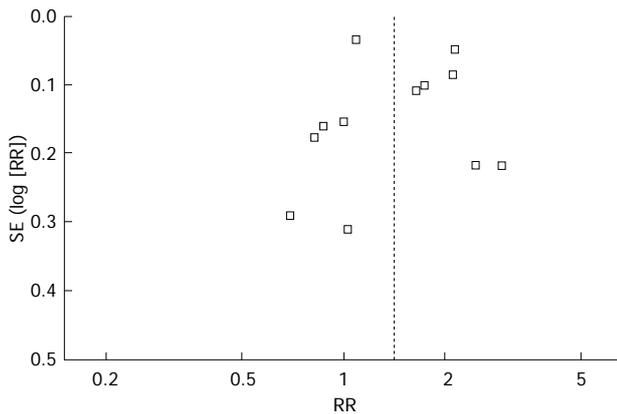


Figure 3 Funnel plot analysis of all the included publications.

between T1DM and a greater risk of developing cancer is equivocal<sup>[38]</sup>, factors other than glucose may play an important role. Besides hyperglycemia, a second hallmark of T2DM is hyperinsulinemia, resulting from insulin resistance in peripheral tissues for many years both before and after diagnosis; in fact, hyperinsulinemia may be the main culprit for cancer development. Insulin is capable of activating insulin-like growth factor (IGF)- I by enhancing hepatic IGF- I synthesis and is also capable of increasing the bioavailability of IGF- I by reducing hepatic production of the IGF-binding proteins<sup>[39,40]</sup>. Enhanced insulin and IGF- I signals through insulin and IGF- I receptors, respectively, promote cell proliferation and growth via multiple cellular signaling cascades<sup>[39-41]</sup>. Indeed, the overexpression of IGFs and the IGF- I receptor was observed in GC tissues<sup>[42,43]</sup>, and increased expression of the IGF- I receptor was correlated with cancer aggressiveness<sup>[44]</sup> or poor survival<sup>[45]</sup>, suggesting a functional insulin-IGF axis in GC.

Third, the etiology of GC is multifactorial and may be associated with several confounding factors such as increased body mass index and *Helicobacter pylori* (*H. pylori*) infection. Visceral fat *per se* contributes to cancer risk<sup>[46]</sup>, and possible underlying molecular mechanisms linking with obesity that foster cancer development have been demonstrated<sup>[39,46,47]</sup>. Accordingly, one recent meta-analysis has revealed that overweight and obesity correlated with GC<sup>[48]</sup>, findings which are consistent with other types of cancer<sup>[39,46,47]</sup>. Regarding *H. pylori* infection, DM patients showed a higher frequency than non DM subjects both in the West<sup>[49]</sup> and in the East<sup>[50]</sup>, and *H. pylori* infection was in turn correlated with insulin resistance<sup>[51]</sup>, suggesting that DM is liable to cause *H. pylori* infection and *vice versa*. Accordingly, GC risk was dramatically increased when DM and *H. pylori* infection coexisted<sup>[21]</sup>.

One novel finding in this study is a positive DM-GC association in both sexes with a more prominent association in females than in males, which contrasts with the male preponderance of GC in the general population. Such a seemingly inverse sex distribution of GC in DM subjects may be attributable to the decreased sex hormone-binding globulin under increased IGF-

I and hyperinsulinemia<sup>[52]</sup>, leading to increased bioavailability of estrogen in both sexes and increased levels of bioavailable testosterone in women but not in men<sup>[53]</sup>. These mechanisms are plausible explanations for an increased risk of hormone-dependent cancers such as breast cancer in female DM patients. Therefore, it can be speculated that the alterations of sex hormones may influence the magnitude of GC risk by gender in DM patients. On the other hand, the present study revealed the increased risk of GC in populations in East Asia but not in the West, findings which are consistent with one previous study<sup>[20]</sup>. These results can be explained partly by the geographical difference in GC risk<sup>[54]</sup>, and partly by the more established screening program in East Asian countries than in the Western countries. This speculation is supported by similar findings of a greater gastric cardia cancer risk in East Asia than in the West among the *H. pylori*-infected patients<sup>[55]</sup>. Interestingly, a similar geographical difference was also observed in the DM-prostate cancer association<sup>[1,2]</sup>.

There are several limitations to this meta-analysis. First, besides obesity and *H. pylori* infection, GC development appear to be confounded by the possible presence of shared cancer-promoting or -preventing factors such as an unhealthy diet (*e.g.*, high salt intake<sup>[56]</sup> or heavy alcohol drinking<sup>[57]</sup>), sedentary lifestyle with lack of physical activity, duration of the DM state, and the consumption of vegetables, fruit<sup>[58]</sup>, and green tea<sup>[59]</sup>. In addition, some diabetes treatments may increase or decrease cancer risk. These confounding factors make it difficult to accurately assess GC risk in DM patients. Therefore, investigation into the actual GC risk in DM patients requires adjustment based on these confounding factors. This is reflected by the significant heterogeneity, which has been also observed in the previous three meta-analyses; thus, further analyses are warranted. A second limitation is that most studies included in this study reported a DM and GC risk without distinction between T1DM and T2DM. Since T1DM is less prevalent than T2DM<sup>[38]</sup>, most patients in this meta-analysis can be regarded as T2DM. However, the DM-GC association should be further elucidated with distinction between the two types since they differ considerably in their metabolic characteristics.

The diversity of DM conditions and cancer biology, as well as the complexity of the potentially contributory mechanisms, preclude a definitive description of the association between DM and cancer risk at present. Although the precise biological mechanisms that might link DM to cancer remain a matter of debate, the recent surge in attempts to explore the relationship between the two diseases has motivated considerable investigation among the clinical and research communities. This meta-analysis suggests that newer, comprehensive approaches must be developed for the treatment of DM patients as a whole rather than as a single disease. However, it is also true that DM patients are less likely to be screened for several types of cancers<sup>[60-62]</sup>, which may be attributable to the patient preference to focus on the treatment of DM

rather than prevention of cancer<sup>[62]</sup>, when DM consumes his/her attention. Clinicians caring for patients with DM should remain alert to GC and minimize the number of missed opportunities for its treatment.

## COMMENTS

### Background

Besides cardiovascular complications, evidence has accumulated that diabetes mellitus (DM) patients are highly predisposed to many types of cancer. Among the cancer subtypes investigated, however, knowledge on the link between gastric cancer (GC) and DM has been insufficient and inconsistent even in previous meta-analyses.

### Research frontiers

Several meta-analyses have been published to investigate the association between DM and GC, however, the results have been inconsistent and varied, from inverse to positive DM-GC associations, indicating that the link between the two diseases has been unclear.

### Innovations and breakthroughs

DM exhibited significantly increased GC risk by 41% overall, and by 90% in females, 24% in males, and 77% in East Asians in subgroup analyses. These findings provide evidence in the current debate concerning the DM-GC association. Furthermore, a larger GC risk in female DM patients than in males was found to be marked.

### Applications

Evidence of a positive DM-GC association, together with the positive link between DM and many other types of cancer, suggest a need for development of newer, comprehensive approaches for the treatment of DM patients as a whole rather than as a single disease. Clinicians caring for DM patients should remain alert to GC and minimize the number of missed opportunities for its treatment.

### Terminology

Advanced glycation end products (AGEs) are proteins or lipids that become glyated after exposure to sugars. AGEs contribute to a variety of microvascular and macrovascular complications by engaging the receptor for advanced glycation end products.

### Peer review

This meta-analysis provides useful information to clinical and research field for establishing comprehensive management to DM patients.

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## Effects of probiotics on nonalcoholic fatty liver disease: A meta-analysis

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### Abstract

**AIM:** To investigate the relationship between the gut-liver axis and nonalcoholic fatty liver disease (NAFLD), we performed a meta-analysis to evaluate the effects of probiotic therapy in NAFLD.

**METHODS:** We searched PubMed, Medline, Embase, Web of Science, the Cochrane Library and Chinese Biomedicine Database for all relevant randomized controlled trials on probiotics in patients with NAFLD/non-alcoholic steatohepatitis (NASH). A statistical analysis was performed using RevMan 5.0 software.

**RESULTS:** Four randomized trials involving 134 NAFLD/NASH patients were included. The results showed that probiotic therapy significantly decreased alanine aminotransferase (ALT), aspartate transaminase (AST),

total-cholesterol (T-chol), high density lipoprotein (HDL), tumor necrosis factor (TNF)- $\alpha$  and homeostasis model assessment of insulin resistance (HOMA-IR) [ALT: weighted mean difference (WMD) -23.71, 95%CI: -33.46--13.95,  $P < 0.00001$ ; AST: WMD -19.77, 95%CI: -32.55--7.00,  $P = 0.002$ ; T-chol: WMD -0.28, 95%CI: -0.55--0.01,  $P = 0.04$ ; HDL: WMD -0.09, 95%CI: -0.16-0.01,  $P = 0.03$ ; TNF- $\alpha$ : WMD -0.32, 95%CI: -0.48--0.17,  $P < 0.0001$ ; HOMA-IR: WMD -0.46, 95%CI: -0.73--0.19,  $P = 0.0008$ ]. However, the use of probiotics was not associated with changes in body mass index (BMI), glucose (GLU) and low density lipoprotein (LDL) (BMI: WMD 0.05, 95%CI: -0.18-0.29,  $P = 0.64$ ; GLU: WMD 0.05, 95%CI: -0.25-0.35,  $P = 0.76$ ; LDL: WMD -0.38, 95%CI: -0.78-0.02,  $P = 0.06$ ).

**CONCLUSION:** Probiotic therapies can reduce liver aminotransferases, total-cholesterol, TNF- $\alpha$  and improve insulin resistance in NAFLD patients. Modulation of the gut microbiota represents a new treatment for NAFLD.

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**Key words:** Probiotics; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Liver function; Insulin resistance; Meta-analysis

**Core tip:** For many decades, researchers have carried out studies on the treatment of nonalcoholic fatty liver disease (NAFLD). However, no firm conclusions have been made regarding the efficacy of various treatments for NAFLD. Here we conducted a meta-analysis of the pooled data from randomized controlled trials to assess the efficacy of probiotic therapies and showed that probiotic therapy significantly decreased alanine aminotransferase, aspartate transaminase, total-cholesterol, high density lipoprotein, tumor necrosis factor- $\alpha$  and homeostasis model assessment of insulin resistance. Thus, probiotics may help to improve liver function, fat metabolism and insulin resistance in NAFLD patients.

Ma YY, Li L, Yu CH, Shen Z, Chen LH, Li YM. Effects of probiotics on nonalcoholic fatty liver disease: A meta-analysis. *World J Gastroenterol* 2013; 19(40): 6911-6918 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6911.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6911>

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by large vacuoles of triglyceride which accumulate in liver cells *via* the process of steatosis in non-alcohol users. The condition can progress into more serious liver diseases, such as nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and more rarely, liver carcinoma<sup>[1]</sup>. It is increasingly recognized as a major cause of liver-related morbidity and mortality. NAFLD is common in Western countries. However, an increase in the prevalence of NAFLD has been observed in China. The underlying mechanisms of disease progression are poorly understood. Diet and lifestyle changes are primary therapies in the management of these patients. Specific pharmacologic treatments for NAFLD/NASH are progressing, such as insulin-sensitizers<sup>[2-4]</sup>, lipid-lowering drugs<sup>[5-6]</sup>, antioxidants<sup>[7-8]</sup>, and anti-tumor necrosis factor (TNF)- $\alpha$  agents<sup>[9-11]</sup>. However, most of these are not licensed therapies for NAFLD, despite the abundance of clinical trials.

Recently, a new treatment strategy using probiotics was proposed. A probiotic is a live microbial culture or cultured dairy product, which plays a fundamentally important role in health and disease<sup>[12-14]</sup>. The human intestinal microbiota is composed of  $10^{13}$ - $10^{14}$  microorganisms whose collective genome contains at least 100 times as many genes as our own genome, representing 500-1000 species in total<sup>[15-16]</sup>. Miele *et al.*<sup>[17]</sup> provided the first evidence that NAFLD in humans was associated with increased intestine permeability, and that this abnormality was related to the increased prevalence of small bowel bacterial overgrowth (SIBO) in these patients. The increased permeability appears to be caused by disruption of intercellular tight junctions in the intestine, and it may play an important role in the pathogenesis of NAFLD. Loguerio *et al.*<sup>[18]</sup> have shown that probiotics may reduce NAFLD liver injury and may improve liver function. Probiotics can inhibit the proliferation of harmful bacteria, reduce SIBO, restore gastrointestinal barrier function and modulate the immune system<sup>[19-21]</sup>, all of which contribute to the improvement of NAFLD.

Therefore, the aim of this study was to conduct a meta-analysis of the pooled data from RCTs to assess the efficacy of probiotic therapies in modifying liver function, fat metabolism and insulin resistance.

## MATERIALS AND METHODS

### Search strategy

We searched Medline, Embase, Web of Science, Chinese

Biomedicine Database and the China Journal Full Text Database with no language restriction. The search terms were: “(NASH or NAFLD or “nonalcoholic steatohepatitis” or “nonalcoholic fatty liver disease” or “fatty liver”) and (probiotic\* or prebiotic\* or synbiocit\* or bifidobacter\* or Lactobacill\* or flora)” and “[“Fatty Liver” (Mesh)] AND “Probiotics”(Mesh)”. We also searched the reference lists of each selected study by hand.

### Inclusion and exclusion criteria

Inclusion criteria were as follows: randomized controlled trials (RCTs) with participants of any sex or ethnic origin with NAFLD/NASH, diagnosed on the basis of radiological/histological evidence of fatty liver. Exclusion criteria were as follows: other causes of hepatic steatosis or steatofibrosis such as hepatitis B, hepatitis C, autoimmune hepatitis, liver decompensation or malignancy, and genetic liver disease such as Wilson’s disease and hemochromatosis.

The trials should have measured at least one of the following items: BMI, ALT, AST, total-cholesterol, LDL, HDL, GLU, TNF- $\alpha$  and HOMA-IR. Studies must have objective outcome measures, otherwise they were excluded from this review.

### Data extraction and methodological quality

Data were abstracted independently by two reviewers and included: author, publication year, study design, population, intervention, duration and outcome. Disagreement was resolved by discussion.

Scored using the Jadad scale, we assessed the quality of the studies by the randomization method, allocation concealment, blinding of outcome assessment and follow-up. All included studies scored  $\geq 4$ .

### Statistical analysis

We analyzed the data using Review Manager 5.0. Dichotomous data were presented as odds ratio with 95%CI. Statistical heterogeneity was measured using the  $\chi^2$  test and the  $I^2$ . A  $\chi^2$  P value  $< 0.05$  was considered to indicate statistically significant heterogeneity. If there was obvious heterogeneity, the random effects model was chosen; otherwise, the fixed effects model was adopted.

## RESULTS

The electronic searches yielded 475 items from Medline, Embase, Web of Science, Chinese Biomedicine Database and the China Journal Full Text Database. Publication dates ranged from 1996 to 2013. After reviewing each publication, we selected 4 original studies (Figure 1).

Table 1 contains specific information on study design, randomization methods, sample size, intervention, duration of treatment and follow-up. Allocation concealment was adequate in three studies. All the studies were double-blind and included a follow-up period. The diagnosis of NAFLD/NASH was confirmed by percutaneous liver biopsy in three studies. All gave detailed

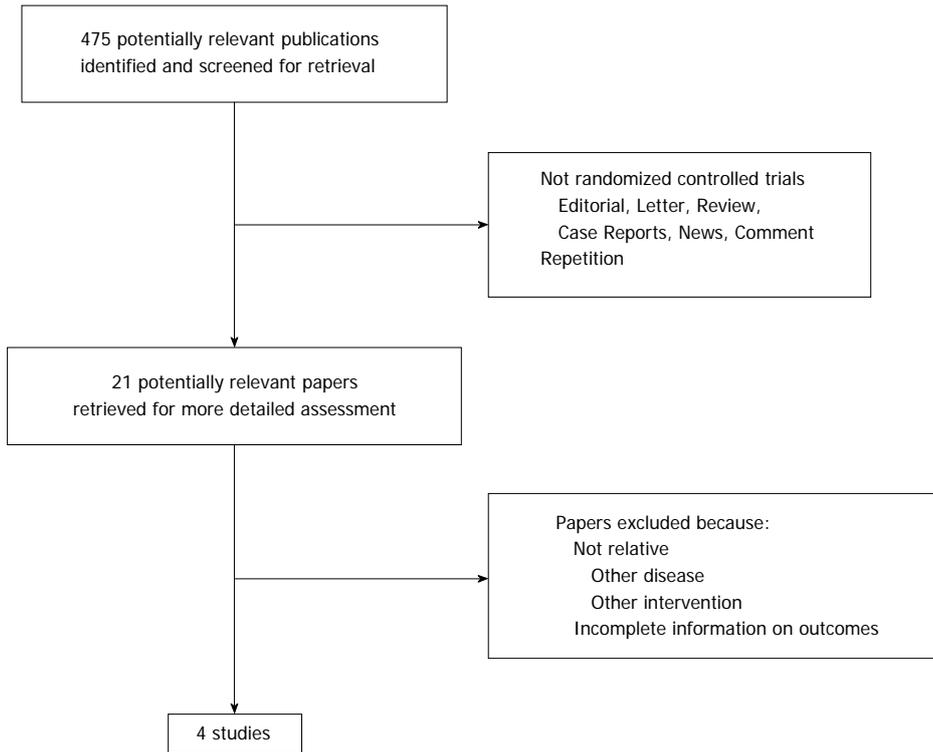


Figure 1 Selection of studies.

**Table 1 Methodological characteristics of the included studies in this meta-analysis**

Ref.	Sample size	Randomization	Blinding	Diagnostic method	Intervention	Duration	Follow-up
Aller <i>et al</i> <sup>[22]</sup>	28 (14/14)	Table of numbers	Double-blind	Histological	Lactobacillus bulgaricus and Streptococcus thermophilus <i>vs</i> placebo	3 mo	Yes
Vajro <i>et al</i> <sup>[23]</sup>	20 (10/10)	Yes	Double-blind	Radiological	Lactobacillus GG <i>vs</i> placebo	8 wk	Yes
Malaguarnera <i>et al</i> <sup>[24]</sup>	66 (34/32)	Computer generated	Double-blind	Histological	Bifidobacterium longum + Fos <i>vs</i> placebo	24 wk	Yes
Wong <i>et al</i> <sup>[25]</sup>	20 (10/10)	Computer generated	Double-blind	Histological	Lepicol probiotic and prebiotic formula <i>vs</i> nothing	6 mo	Yes

baseline information. The main characteristics of the patients included in the two groups were well matched in all RCTs.

All four RCTs<sup>[22-25]</sup> reported on BMI, but did not show a significant difference in the experimental group compared with the control group [weighted mean difference (WMD) 0.05, 95%CI: -0.18-0.29, *P* = 0.64]. Significant homogeneity was observed among the studies (*I*<sup>2</sup> = 0%, *P* = 0.77) (Figure 2A).

Four RCTs<sup>[22-25]</sup> assessed the effect of probiotics on the level of serum ALT and showed a significant difference between patients treated with probiotics compared with those treated with placebo (WMD -23.71, 95%CI: -33.46--13.95, *P* < 0.00001). The included studies were homogeneous (*I*<sup>2</sup> = 0%, *P* = 0.72) (Figure 2B).

Three RCTs<sup>[22,24,25]</sup> analyzed the effect of probiotics on AST and T-chol in NAFLD/NASH patients compared with placebo. Probiotics had a significantly better effect on normalizing AST and T-chol (AST: WMD -19.77, 95%CI: -32.55--7.00, *P* = 0.002; T-chol: WMD -0.28, 95%CI: -0.55--0.01, *P* = 0.04). The included studies on AST were not homogeneous (*I*<sup>2</sup> = 56%, *P* = 0.1), while the studies on T-chol were significantly homoge-

neous (*I*<sup>2</sup> = 0%, *P* = 0.75) (Figure 2C, D).

Three RCTs<sup>[22,24,25]</sup> reported the effects of probiotics on LDL, HDL and GLU in patients with NAFLD/NASH compared with placebo. Probiotics had a significantly better effect in reducing HDL (WMD -0.09, 95%CI: -0.16-0.01, *P* = 0.03), but no significant difference in reducing LDL and GLU (LDL: WMD -0.38, 95%CI: -0.78-0.02, *P* = 0.06; GLU: WMD 0.05, 95%CI: -0.25-0.35, *P* = 0.76). The included studies were homogeneous (LDL: *I*<sup>2</sup> = 47%, *P* = 0.15; HDL: *I*<sup>2</sup> = 0%, *P* = 0.53; GLU: *I*<sup>2</sup> = 0%, *P* = 0.84) (Figure 2E, F, G).

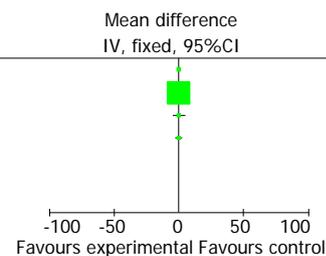
Three RCTs<sup>[22-24]</sup> provided sufficient data to compare the effects of probiotics with those of placebo and showed a statistically significant effect for TNF-α in NAFLD/NASH patients (WMD -0.32, 95%CI: -0.48--0.17, *P* < 0.0001). Significant homogeneity was observed among the studies (*I*<sup>2</sup> = 0%, *P* = 0.56) (Figure 2H).

Only two RCTs<sup>[22,24]</sup> reported the effects of probiotics on HOMA-IR in NAFLD/NASH patients. There was a significant reduction in HOMA-IR in NAFLD/NASH patients in the experimental group compared with the control group (WMD -0.46, 95%CI: -0.73--0.19, *P* =

**A** BMI

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-0.9	1.68	34	-1.3	1.89	32	7.1%	0.40 (-0.46, 1.26)
Pietro Vajro 2011	-0.08	0.3	10	-0.12	0.25	10	90.5%	0.04 (-0.20, 0.28)
R. ALLER 2007	0.9	4.7	14	0.6	5.9	14	0.3%	0.30 (-3.65, 4.25)
Wong 2013	-1	2.3	10	-0.5	1.1	10	2.1%	0.50 (-2.08, 1.08)
Total (95%CI)			68			66	100.0%	0.05 (-0.18, 0.29)

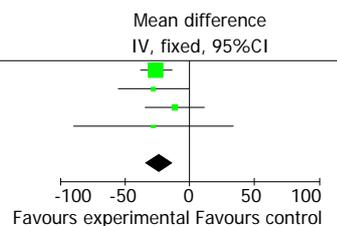
Heterogeneity:  $\chi^2 = 1.11$ ,  $df = 3$  ( $P = 0.77$ );  $I^2 = 0\%$   
 Test for overall effect:  $Z = 0.47$  ( $P = 0.64$ )



**B** The level of serum ALT

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-63.9	23.1	34	-38	26	32	67.1%	-25.90 (-37.79, -14.01)
Pietro Vajro 2011	-30.2	32.4	10	-2	29.9	10	12.7%	-28.20 (-55.53, -0.87)
R. ALLER 2007	-7.3	28.6	14	4.1	34.1	14	17.5%	-11.40 (-34.71, 11.91)
Wong 2013	-26	91	10	2	41	10	2.5%	-28.00 (-89.86, 33.86)
Total (95%CI)			68			66	100.0%	-23.71 (-33.46, -13.95)

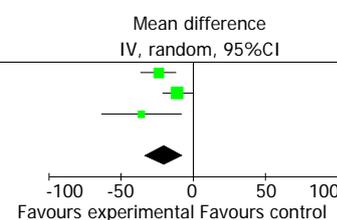
Heterogeneity:  $\chi^2 = 1.32$ ,  $df = 3$  ( $P = 0.72$ );  $I^2 = 0\%$   
 Test for overall effect:  $Z = 4.76$  ( $P < 0.00001$ )



**C** The level of serum AST

Study or subgroup	Experimental			Control			Weight	Mean difference IV, random, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-69.6	26.51	34	-45.9	23.98	32	39.8%	-23.70 (-35.88, -11.52)
R. ALLER 2007	-5.7	14.44	14	4.7	13.48	14	44.3%	-10.40 (-20.75, -0.05)
Wong 2013	-13	31	10	23	32	10	15.9%	-36.00 (-63.61, -8.39)
Total (95%CI)			58			56	100.0%	-19.77 (-32.55, -7.00)

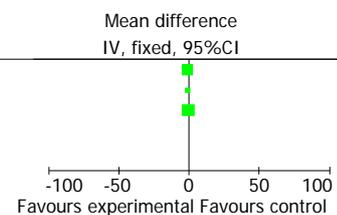
Heterogeneity:  $\tau^2 = 68.10$ ,  $\chi^2 = 4.53$ ,  $df = 2$  ( $P = 0.10$ );  $I^2 = 56\%$   
 Test for overall effect:  $Z = 3.03$  ( $P = 0.002$ )



**D** The level of serum T-chol

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-0.6	0.88	34	-0.2	0.83	32	43.1%	-0.40 (-0.81, 0.01)
R. ALLER 2007	0.16	1.06	14	0.31	1.2	14	10.4%	-0.15 (-0.99, 0.69)
Wong 2013	0	0.4	10	0.2	0.5	10	46.5%	-0.20 (-0.60, 0.20)
Total (95%CI)			58			56	100.0%	-0.28 (-0.55, -0.01)

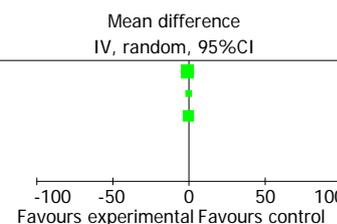
Heterogeneity:  $\chi^2 = 0.57$ ,  $df = 2$  ( $P = 0.75$ );  $I^2 = 0\%$   
 Test for overall effect:  $Z = 2.03$  ( $P = 0.04$ )



**E** The level of serum LDL

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-0.84	0.69	34	-0.18	0.69	32	47.2%	-0.66 (-0.99, -0.33)
R. ALLER 2007	0.29	1.21	14	0.29	0.92	14	18.4%	0.00 (-0.80, 0.80)
Wong 2013	0.1	0.6	10	0.3	0.5	10	34.4%	-0.20 (-0.68, 0.28)
Total (95%CI)			58			56	100.0%	-0.38 (-0.78, 0.02)

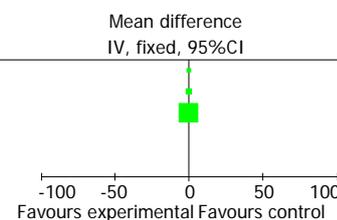
Heterogeneity:  $\tau^2 = 0.06$ ,  $\chi^2 = 3.78$ ,  $df = 2$  ( $P = 0.15$ );  $I^2 = 47\%$   
 Test for overall effect:  $Z = 1.88$  ( $P = 0.06$ )



**F** The level of serum HDL

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	0.13	0.73	34	0.02	0.74	32	4.8%	0.11 (-0.24, 0.46)
R. ALLER 2007	0	0.3	14	0.08	0.2	14	16.9%	-0.08 (-0.27, 0.11)
Wong 2013	0	0.1	10	0.1	0.1	10	78.3%	-0.10 (-0.19, -0.01)
Total (95%CI)			58			56	100.0%	-0.09 (-0.16, -0.01)

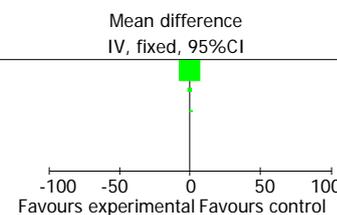
Heterogeneity:  $\chi^2 = 1.27$ ,  $df = 2$  ( $P = 0.53$ );  $I^2 = 0\%$   
 Test for overall effect:  $Z = 2.19$  ( $P = 0.03$ )



**G** The level of serum GLU

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-0.65	0.65	34	-0.68	0.66	32	90.5%	0.03 (-0.29, 0.35)
R. ALLER 2007	-0.07	1.5	14	-0.13	1.61	14	6.8%	0.06 (-1.09, 1.21)
Wong 2013	0.8	2.9	10	0.2	0.7	10	2.6%	0.60 (-1.25, 2.45)
Total (95%CI)			58			56	100.0%	0.05 (-0.25, -0.35)

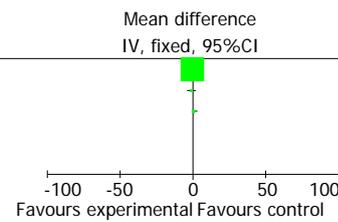
Heterogeneity:  $\chi^2 = 0.36$ ,  $df = 2$  ( $P = 0.84$ );  $I^2 = 0\%$   
 Test for overall effect:  $Z = 0.31$  ( $P = 0.76$ )



**H** The level of serum TNF- $\alpha$

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-0.45	0.34	34	-0.12	0.29	32	99.4%	-0.33 (-0.48, -0.18)
Pietro Vajro 2011	-2.32	6.02	10	-1.4	5.09	10	0.1%	-0.92 (-5.81, 3.97)
R. ALLER 2007	0.62	3	14	-0.14	2.48	14	0.6%	0.76 (-1.28, 2.80)
Total (95%CI)			58			56	100.0%	-0.32 (-0.48, -0.17)

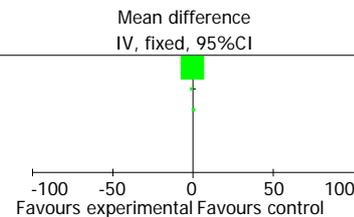
Heterogeneity:  $\chi^2 = 1.15$ ,  $df = 2$  ( $P = 0.56$ );  $I^2 = 0\%$   
 Test for overall effect:  $Z = 4.19$  ( $P < 0.0001$ )



**I** HOMA

Study or subgroup	Experimental			Control			Weight	Mean difference IV, fixed, 95%CI
	Mean	SD	Total	Mean	SD	Total		
Malaguarnera 2012	-1.1	0.52	34	-0.64	0.6	32	98.5%	-0.46 (-0.73, -0.19)
R. ALLER 2007	-0.3	2.51	14	0.1	3.31	14	1.5%	-0.40 (-2.58, 1.78)
Total (95%CI)			48			46	100.0%	-0.46 (-0.73, -0.19)

Heterogeneity:  $\chi^2 = 0.00$ ,  $df = 1$  ( $P = 0.96$ );  $I^2 = 0\%$   
 Test for overall effect:  $Z = 3.34$  ( $P = 0.0008$ )



**Figure 2** Forest plot of the effects of probiotics in patients with nonalcoholic fatty liver disease. A: BMI; B: The level of serum ALT; C: The level of serum AST; D: The level of serum T-chol; E: The level of serum LDL; F: The level of serum HDL; G: The level of serum GLU; H: The level of serum TNF- $\alpha$ ; I: HOMA. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate transaminase; T-chol: total-cholesterol; LDL: Low density lipoprotein; HDL: High density lipoprotein; GLU: Glucose; HOMA: Homeostasis model assessment.

0.0008). Significant homogeneity was observed among the studies ( $I^2 = 0\%$ ,  $P = 0.96$ ) (Figure 2I).

**DISCUSSION**

NAFLD is a relevant issue in public health due to its epidemiologic burden. The prevalence of NAFLD has apparently increased in proportion to the increasing incidence of obesity in both adults and children<sup>[26]</sup>. NAFLD is closely associated with obesity and insulin resistance, and is now recognized to represent the hepatic manifestation of the metabolic syndrome. At present, there is no registered drug for the treatment of NAFLD. Although lifestyle intervention is often advocated<sup>[27-28]</sup>, it is difficult to maintain. In 2009, Socha *et al*<sup>[29]</sup> performed a meta-analysis of the pharmacological interventions for NAFLD in adults and children, including pioglitazone, vitamin E, ursodeoxycholic acid, probucol, N-acetylcysteine, and low-dose carnitine. However, he was unable to draw firm conclusions on the efficacy of the various treatments for NAFLD. In 2011, Musso *et al*<sup>[30]</sup> found that weight loss improved liver histology and the cardio-metabolic profile, as did pioglitazone. It is also important to explore new treatment strategies.

It is well known that liver and intestine have the same origin in embryology the foregut. In addition, the liver continuously receives blood from the gut through the portal system. Therefore, there is a close relationship between the intestine and liver. Evidence has shown that SIBO is present in 50% of patients with non-alcoholic steatosis<sup>[17,31]</sup>. High-fat diet-induced obesity is associated with changes in the composition of intestinal bacteria in rats<sup>[32-33]</sup> and in humans<sup>[34]</sup>. Therefore, changes in the composition of the intestinal bacterial content may be associated with NAFLD or obesity.

Intestinal bacteria may be involved in the etiology of NAFLD by enhancing intestinal permeability<sup>[35]</sup>, direct

activation of inflammatory cytokines *via* release of lipopolysaccharide (LPS) and favoring absorption of endotoxins<sup>[36]</sup>. Endotoxins activate Kupffer cells in the liver and increase the production of TNF- $\alpha$  and IL-6, which contributes to the onset of liver fibrosis<sup>[31,37-38]</sup>. Furthermore, a complex mechanism involving extensive lipid accumulation, systemic inflammation, oxidative stress, and insulin resistance causes cytotoxicity and exacerbated hepatopathy<sup>[39-40]</sup>.

Serum ALT and AST levels are well-recognized clinical markers of liver damage and may be involved in the pathogenesis of NAFLD. Cholesterol is also a risk factor for NAFLD. Liver damage can lead to elevated cholesterol or reduced HDL in the blood. TNF- $\alpha$  is secreted directly by hepatocytes and Kupffer cells in the liver<sup>[41]</sup>. Many studies have shown a relationship between TNF- $\alpha$  expression and NAFLD<sup>[42-43]</sup>. Assessment of insulin resistance by HOMA-IR has been widely utilized in clinical studies of NAFLD<sup>[44-45]</sup>. In four RCTs, ALLER, Wong *et al*<sup>[25]</sup> reported that probiotics improved liver aminotransferase levels in patients with NAFLD, while Malaguarnera concluded that probiotics reduced TNF- $\alpha$ , serum AST levels and HOMA-IR. Our meta-analysis showed that probiotics significantly reduced ALT, AST, T-chol, TNF- $\alpha$  and HOMA-IR, which are all related to the process, development and consequences of NAFLD. However, the level of HDL was significantly increased in the placebo treatment compared with probiotic treatment, which was contrary to expectation. It is possible that the elevation in HDL requires long-term treatment or there are other mechanisms which have not been explored.

The change in cholesterol level in our study should be emphasized, as Gilliland *et al*<sup>[46]</sup> in the early 1990s found that regular consumption of probiotics reduced cholesterol levels. Over several decades, more and more researchers confirmed that probiotics can lead to a de-

crease in serum cholesterol in animals and humans<sup>[47-50]</sup>. However, these RCTs did not report the positive effects of probiotics on reducing cholesterol in NAFLD/NASH patients, while the findings of the present meta-analysis supported the reduction of cholesterol in NAFLD/NASH patients. From this meta-analysis, we can conclude that probiotics have positive effects in patients with NAFLD/NASH.

Of the four RCTs included in this meta-analysis, the studied probiotics included lactobacillus, bifidobacterium and streptococcus. Two studies also determined the effect of probiotics combined with fructo-oligosaccharides in NAFLD<sup>[24,25]</sup>. Bifidobacteria colonize the intestinal tract soon after birth and are the major components of the microbial barrier in healthy humans. Bifidobacteria produce a range of beneficial effects on host health<sup>[51-52]</sup>. Lactobacilli and streptococcus are also beneficial, although they are present at much lower levels in the human colon<sup>[52]</sup>. Probiotics have been shown to enhance the barrier function of epithelial cells<sup>[53]</sup> and decrease intestinal permeability and endotoxemia in patients with liver disease<sup>[54]</sup>. At the same time, probiotics can also influence host metabolism in several other ways, such as regulation of energy extraction from nutrients and modulation of genes involved in substrate metabolism<sup>[55]</sup>. A prebiotic is a nondigestible food ingredient. Due to the general properties of prebiotics, they can influence the growth, activity and metabolites of probiotics<sup>[56]</sup>. Fructo-oligosaccharides are now becoming increasingly popular due to their prebiotic effects. They can be fermented by bifidobacteria and lactobacilli<sup>[57]</sup>. Fructo-oligosaccharides can lead to bifidobacteria becoming the dominant species in the large bowel<sup>[58]</sup> and may help to control or reduce the growth of harmful bacteria<sup>[59]</sup>. In animal models, treatment with oligofructose reduced adipose tissue inflammation, oxidative stress and led to an improvement in glucose tolerance and to a reduction in body weight, which were beneficial in patients with NAFLD<sup>[60]</sup>. In conclusion, probiotics and prebiotics are important mediators of diet-induced metabolic disturbances in NAFLD.

There are several limitations to this review. It is well known that liver histology is the gold standard for NAFLD/NASH. Although ultrasonography is reasonably accurate, it cannot identify fatty infiltration of the liver below a threshold of 30%. In our review, three RCTs used liver histological response as an outcome index evaluating the effectiveness of probiotics in the treatment of NAFLD. Regrettably, only one RCT had post-treatment histology results. The diagnostic criteria for NAFLD in another trial included increased ultrasonographic bright liver. Three trials included patients aged 18-70 years, while one trial included children. The researchers ignored the dietary restrictions, exercise and physical activities as in almost all studies they were not described. The sample sizes in some trials, as well as the number of trials for some comparisons, were small. Existing data are difficult to reconcile, given the use of different

strains, dosages and duration of treatment.

## COMMENTS

### Background

The prevalence of and mortality due to nonalcoholic fatty liver disease (NAFLD) are increasing worldwide. Diet and lifestyle changes are primary therapies in the management of NAFLD patients. However, most drug therapies are not licensed for NAFLD. Recent evidence suggests that malfunction of the gut-liver axis contributes to hepatic damage in rats and humans with NAFLD, and probiotics play a fundamentally important role in health and disease. Thus, it was necessary to conduct a meta-analysis to assess the effects of probiotics on liver function, fat metabolism and insulin resistance in NAFLD patients.

### Research frontiers

A probiotic is a live microbial culture or cultured dairy product and the human intestinal microbiota is composed of  $10^{13}$ - $10^{14}$  microorganisms. The gut-liver axis indicates that changes in the composition of the intestinal bacterial content are associated with NAFLD. Most therapies are not licensed for the prevention of NAFLD. Therefore, a research hotspot is whether treatment with probiotics is effective in patients with NAFLD.

### Innovations and breakthroughs

In 2009, Socha *et al* performed a meta-analysis of pharmacological interventions for NAFLD in adults and children, including pioglitazone, vitamin E, ursodeoxycholic acid, probucol, N-acetylcysteine, and low-dose carnitine. However, he was unable to draw firm conclusions on the efficacy of various treatments for NAFLD and he did not study the effect of probiotics. Research on probiotics in rats is popular. However, there have only been a few large randomized controlled trials (RCTs), and the results were inconsistent. Therefore, the aim of this study was to conduct a meta-analysis of the pooled data from RCTs to assess the efficacy of probiotic therapies in modifying liver function, fat metabolism and insulin resistance in patients with NAFLD/nonalcoholic steatohepatitis.

### Applications

Probiotic therapies can reduce liver aminotransferase levels, serum cholesterol and tumor necrosis factor- $\alpha$  and improve insulin resistance in patients with NAFLD. Thus, modulation of the gut microbiota using probiotics may represent a new method of treating or preventing NAFLD.

### Terminology

NAFLD is characterized by large vacuoles of triglyceride which accumulate in liver cells via the process of steatosis in non-alcohol users. The condition can progress into more serious liver diseases, such as nonalcoholic steatohepatitis, liver fibrosis, cirrhosis, and more rarely, liver carcinoma. A probiotic is a live microbial culture or cultured dairy product, which plays a fundamentally important role in health and disease. The human intestinal microbiota is composed of  $10^{13}$ - $10^{14}$  microorganisms whose collective genome contains at least 100 times as many genes as our own genome, representing 500-1000 species in total.

### Peer review

This meta-analysis is an interesting revision on the present knowledge on probiotics and NAFLD but again introduces the difficulty in obtaining unequivocal correlations between biochemical changes and pharmacological treatment or lifestyle modifications, including the inclusion of probiotics in diet. This meta-analysis suggests that liver inflammatory markers become significantly reduced with the use of probiotics and this has been interpreted as indirect evidence of the effect on inflammation and liver damage by the intervention. Nevertheless, the meta-analysis concludes that many of the data analyzed remain without modification on pooled analysis with the inclusion of probiotics. All of these data make it difficult to assess the real effect of probiotics on NAFLD. Nevertheless, the data obtained by this meta-analysis are interesting since they demonstrate the complexity of the factors which may influence the development of NAFLD.

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## Restrictive vs liberal transfusion for upper gastrointestinal bleeding: A meta-analysis of randomized controlled trials

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### Abstract

**AIM:** To compare the outcome of upper gastrointestinal bleeding (UGIB) between patients receiving restrictive and liberal transfusion.

**METHODS:** PubMed, EMBASE, and Cochrane Library databases were employed to identify all relevant randomized controlled trials regarding the outcome of UGIB after restrictive or liberal transfusion. Primary outcomes were death and rebleeding. Secondary outcomes were length of hospitalization, amount of blood transfused, and hematocrit and hemoglobin at discharge or after expansion.

**RESULTS:** Overall, 4 papers were included in this

meta-analysis. The incidence of death was significantly lower in patients receiving restrictive transfusion than those receiving liberal transfusion (OR: 0.52, 95%CI: 0.31-0.87,  $P = 0.01$ ). The incidence of rebleeding was lower in patients receiving restrictive transfusion than those receiving liberal transfusion, but this difference did not reach any statistical significance (OR: 0.26, 95%CI: 0.03-2.10,  $P = 0.21$ ). Compared with those receiving liberal transfusion, patients receiving restrictive transfusion had a significantly shorter length of hospitalization (standard mean difference: -0.17, 95%CI: -0.30--0.04,  $P = 0.009$ ) and a significantly smaller amount of blood transfused (standard mean difference: -0.74, 95%CI: -1.15--0.32,  $P = 0.0005$ ) with a lower hematocrit and hemoglobin level at discharge or after expansion.

**CONCLUSION:** Restrictive transfusion should be employed in patients with UGIB.

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**Key words:** Upper gastrointestinal bleeding; Blood transfusion; Meta-analysis; Randomized controlled trial

**Core tip:** Current international consensus recommends restrictive transfusion for upper gastrointestinal bleeding. However, this recommendation is largely based on expert opinions. We have performed the present meta-analysis of randomized controlled trials, which potentially supported the superiority of restrictive over liberal transfusion for upper gastrointestinal bleeding.

Wang J, Bao YX, Bai M, Zhang YG, Xu WD, Qi XS. Restrictive vs liberal transfusion for upper gastrointestinal bleeding: A meta-analysis of randomized controlled trials. *World J Gastroenterol* 2013; 19(40): 6919-6927 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6919.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6919>

## INTRODUCTION

Acute upper gastrointestinal bleeding is a common cause for emergency hospitalization with a relatively high annual incidence of 50-200/100000 population and a mortality of 10%-30%<sup>[1-3]</sup>. Red blood cell transfusion is often required in such patients due to the reduction of tissue perfusion after acute blood loss<sup>[4-6]</sup>. Current international consensus on the management of upper gastrointestinal bleeding recommends that the threshold for initiating blood transfusion is a hemoglobin level of 70 g/L or less in patients with nonvariceal upper gastrointestinal bleeding<sup>[5]</sup> and a hemoglobin level of 80 g/L or less in patients with variceal bleeding<sup>[6]</sup>. However, these recommendations are largely based on expert opinions or international guidelines regarding transfusion requirement in critically ill patients without upper gastrointestinal bleeding<sup>[7-9]</sup>. Accordingly, the grade of evidence for these recommendations is low<sup>[5,6]</sup>.

A previous Cochrane Collaboration systematic review of three randomized controlled trials has shown a tendency in decreasing the mortality of patients with upper gastrointestinal bleeding after restrictive transfusion<sup>[10,11]</sup>. But a small number of participants and a high proportion of missing data limit the significance of these findings<sup>[10,11]</sup>. These authors concluded that their review might not provide useful data regarding outcomes following red blood cell transfusion for acute upper gastrointestinal bleeding<sup>[10,11]</sup>. Recently, several large-scale observational studies demonstrated that blood transfusion after non-variceal upper gastrointestinal bleeding might increase the rate of mortality and rebleeding<sup>[12,13]</sup>. More recently, a large and well-organized randomized controlled trial showed a significant benefit of restrictive transfusion strategy in improvement of outcome in patients with upper gastrointestinal bleeding<sup>[14]</sup>.

Herein, we performed an updated meta-analysis of randomized controlled trials to compare the outcome of upper gastrointestinal bleeding between patients treated with restrictive and liberal transfusion. Primary outcomes were death and rebleeding. Secondary outcomes were length of hospitalization, amount of blood transfused, and hematocrit and hemoglobin at discharge or after expansion.

## MATERIALS AND METHODS

This work was performed according to the PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions<sup>[15]</sup>.

### Eligibility criteria

(1) randomized controlled trials were included, if they compared the outcome of upper gastrointestinal bleeding between patients treated with restrictive and liberal blood transfusion; (2) no publication date, publication language, or publication status was restricted; (3) basic studies were excluded; (4) comments, editorials, or letters were excluded; (5) introductions for Cochrane groups were excluded; (6) narrative reviews, systematic reviews, or meta-analyses were excluded; (7) study design reports, case reports, non-

comparative case series were excluded; (8) non-randomized comparative studies were excluded; and (9) randomized controlled trials unrelated to our topics were excluded.

### Literature selection

Studies were identified using a search strategy in the PubMed, EMBASE, and Cochrane Library databases. Search items were listed as follows: (“gastrointestinal” [All Fields] OR “digestive” [All Fields] OR “peptic” [All Fields] OR “alimentary tract” [All Fields] OR “esophageal” [All Fields] OR “esophagus” [All Fields] OR “gastric” [All Fields] OR “stomach” [All Fields] OR “duodenal” [All Fields] OR “duodenum” [All Fields]) AND (“hemorrhage” [All Fields] OR “haemorrhage” [All Fields] OR “bleeding” [All Fields] OR “bleed” [All Fields] OR “melena” [All Fields] OR “melaema” [All Fields] OR “hematemesis” [All Fields] OR “haematemesis” [All Fields]) AND (“transfusion” [All Fields] AND (“blood” [All Fields] OR “red cell” [All Fields]) AND (“randomized” [All Fields] OR “randomized” [All Fields] OR “randomly” [All Fields])). The last search was performed on January 5, 2013. Study eligibility was independently judged by two authors. In cases of disagreement between the two authors, they would be discussed with another author. When two or more studies were conducted by one affiliation, only the studies with more complete data and more extensive interval of enrollment were included in the meta-analysis.

### Data extraction

We developed a data extraction sheet, including the authors, journal, publication year, whether articles were published in peer-reviewed journals or not, regions where the study was conducted, period of enrollment, study design, target population, study endpoints, eligibility criteria, sample size, and demographic data (age and sex), source of upper gastrointestinal bleeding, hematocrit, and hemoglobin of two groups. Data were independently extracted by two authors. In cases of disagreement between the two authors, they would be discussed with another author. We used Google to translate the information in the non-English language full-texts.

### Assessment of study quality

Quality of included randomized controlled trials was scored by Jadad composite scale<sup>[16,17]</sup>, in which the description of randomization, blinding, and withdrawals were assessed. The quality scale ranged from 0 to 5 points. If the score was 2 or less, the quality of the study would be considered lower. If the score was beyond 2, the quality of the study would be high.

### Statistical analysis

Data were collected, using Microsoft Office Excel 2003. For the dichotomous variables (*i.e.*, incidence of death and rebleeding), number of events and total patients were extracted from these included studies. Then, OR with 95%CI was calculated. For the continuous variables (*i.e.*, length of hospitalization, amount of blood transfu-

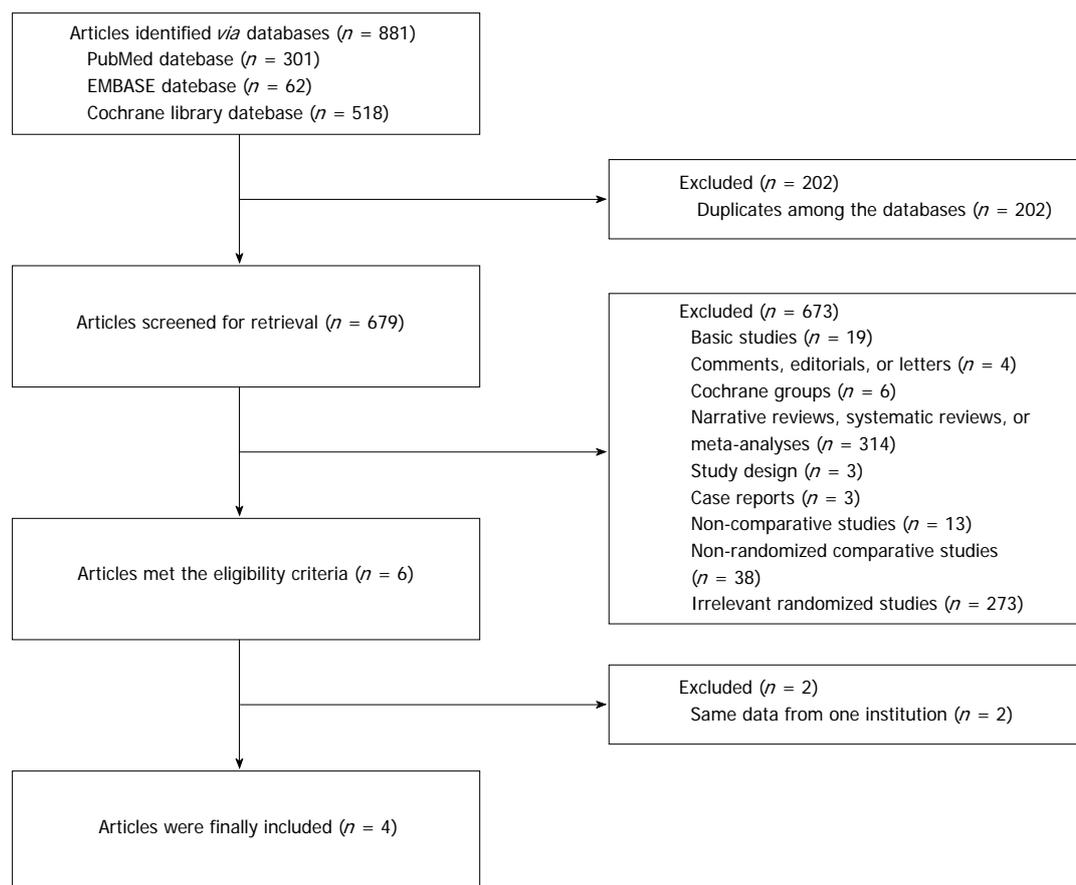


Figure 1 Flowchart of study inclusion.

sion, and hematocrit and hemoglobin), mean value and standard deviation were extracted from these included studies. Then, standard mean difference with 95%CI was calculated. Finally, the OR and standard mean difference of each study were pooled, using both fixed-effects (Mantel-Haenszel method)<sup>[18]</sup> and random-effects model (DerSimonian-Laird method)<sup>[19]</sup>. When heterogeneity was not significant, the pooled data using fixed-effects model were considered appropriate. When significant heterogeneity was observed, only the pooled data using random-effects model were considered appropriate. Heterogeneity between studies was assessed by using the  $I^2$  statistic ( $I^2 > 50\%$  was considered as having substantial heterogeneity) and the  $\chi^2$  test ( $P < 0.10$  was considered to represent significant statistical heterogeneity)<sup>[20]</sup>. Funnel plots were not performed due to a small number of included studies. Sensitivity analyses were done to assess the reliability of meta-analysis. All analyses were conducted using the statistical package Review Manager version 5.1 (Copenhagen, The Nordic Cochrane Center, The Cochrane Collaboration, 2011).

## RESULTS

### Literature selection

Overall, 881 papers were initially identified by the search strategy. Among them, six papers met eligibility crite-

ria<sup>[14,21-25]</sup>. Of note, three papers were reported by the same one affiliation<sup>[14,21-22]</sup>. Among them, two papers with a smaller number of patients were excluded<sup>[21,22]</sup>. Thus, four randomized controlled trials were finally included in the meta-analysis<sup>[14,23-25]</sup> (Figure 1), in which 982 patients with upper gastrointestinal bleeding were treated with restrictive or liberal blood transfusion.

### Description of these included studies

All of four randomized controlled trials were single-center studies, and were published in peer-reviewed journals between 1986 and 2013 (Table 1). Three randomized controlled trials were published in three English-language priority journals<sup>[14,24,25]</sup>, and another one was published in one Spanish-language journal<sup>[23]</sup>. Target population was patients with upper gastrointestinal bleeding from variceal or non-variceal source. Of note, a total of 60 patients were randomized in one study<sup>[23]</sup>, but only 27 patients were finally observed (restrictive transfusion,  $n = 14$ , and liberal transfusion,  $n = 13$ ). The detailed eligibility criteria of these studies were described in Table 2. The baseline characteristics were comparable between the two groups (Table 3).

### Quality of these included studies

In the four studies, double-blinding technique was not feasible due to the nature of interventions. Two studies were scored as 3 points and considered to be of higher

**Table 1 Characteristics of included studies**

Ref.	Design	Regions	Target population	Intervention	Outcome measures
Blair <i>et al</i> <sup>[25]</sup>	Single center RCT	London, United Kingdom	Acute severe UGIB; no esophageal varices	Early transfusion group (liberal transfusion): at least 2 units of blood No transfusion group (restrictive transfusion): no blood transfusion unless hemoglobin fell below 8 g/dL or they were shocked	Observed results: coagulation results (impedance clotting time, kaolin cephalin clotting time); haematological results (hematocrit); eventual blood transfused; number of rebleed; number of death
Elizalde <i>et al</i> <sup>[24]</sup>	Single center RCT	Barcelona, Spain	Liver cirrhosis with an acute variceal bleeding episode	PRC group (liberal transfusion): 2 units of packed red cells PPL group (restrictive transfusion): 500 mL of a 5% plasma protein solution	Observed items: hemodynamic measurements (cardiopulmonary pressures, cardiac output, wedged and free hepatic venous pressures, mean arterial blood pressure, systemic vascular resistance); hormonal measurements (plasma renin activity, aldosterone levels, norepinephrine); rheological parameters (plasma volume, blood viscosity)
Villarejo <i>et al</i> <sup>[23]</sup>	Single center RCT	Buenos Aires, Argentina	Acute digestive hemorrhage with stable haemodynamics	Treatment group (restrictive transfusion): patients underwent normovolemic hemodilution with crystalloid solutions, and the hematocrit value was maintained as $\geq 21\%$ and $< 28\%$ ; red cell transfusion was given if angina, shock, hemodynamic instability, or hematocrit $< 21\%$ Control group (liberal transfusion): the target of transfusion in these patients was the hematocrit value of $\geq 28\%$	Observed results: organ failure, hospital stay, APACHE II score, red cell transfused, hematocrit, haemoglobin
Villanueva <i>et al</i> <sup>[14]</sup>	Single center RCT	Barcelona, Spain	Upper gastrointestinal bleeding	Liberal transfusion: the hemoglobin threshold for transfusion was 9 g/dL, with a target range for the post-transfusion hemoglobin level of 9-11 g/dL Restrictive transfusion: the hemoglobin threshold for transfusion was 7 g/dL, with a target range for the post-transfusion hemoglobin level of 7-9 g/dL	Primary endpoints: the rate of death from any cause within the first 45 d Secondary endpoints: the rate of further bleeding and the rate of in-hospital complications

PPL: Plasma protein solution; PRC: Packed red cells; RCT: Randomized controlled trial; UGIB: Upper gastrointestinal bleeding.

**Table 2 Eligibility criteria of included studies**

Ref.	Eligibility criteria
Blair <i>et al</i> <sup>[25]</sup>	Consecutive patients with acute severe upper gastrointestinal haemorrhage were prospectively randomized on arrival to receive during their first 24 h in hospital Only known cases of oesophageal varices were excluded as they frequently have abnormal coagulation due to liver disease
Elizalde <i>et al</i> <sup>[24]</sup>	The study population consisted of patients with cirrhosis of the liver admitted for the management of an acute variceal bleeding episode Only patients in whom hemostasia had been achieved within the previous 24-72 h by means of endoscopic sclerotherapy, and who were still anemic (hematocrit $< 30\%$ ) and normovolemic as defined by clinical parameters (systolic pressure $> 100$ mmHg, right atrial pressure $> 2$ cm H <sub>2</sub> O, heart rate $< 100$ beats/min, and urine output $> 0.5$ mL/kg per hour) were eligible for the study Age $< 18$ or $> 80$ yr, renal failure as defined as serum creatinine $> 2$ mg/dL, portal thrombosis, diffuse or multinodular hepatocellular carcinoma, arterial hypertension, peripheral vasculopathy, previous surgical or transjugular intrahepatic portosystemic shunt, bacterial infection, and use of vasoactive drugs to prevent or treat portal hypertension-related bleeding were considered exclusion criteria
Villarejo <i>et al</i> <sup>[23]</sup>	Inclusion criteria: acute high digestive haemorrhage with stable haemodynamics and any aetiology; age $> 15$ yr old Exclusion criteria: history of angina; shock not responsive to volume expansion; requirement of surgery; established renal insufficiency; poliglobulina; bleeding diathesis; acute or chronic liver dysfunction; chronic anemia; pregnancy; sepsis; acute or chronic respiratory failure; haematocrit $< 20\%$ on admission; religious objection to transfusion
Villanueva <i>et al</i> <sup>[14]</sup>	Patients older than 18 yr of age who had hematemesis (or bloody nasogastric aspirate), melena, or both, as confirmed by the hospital staff, were considered for inclusion Patients were excluded if they declined to undergo a blood transfusion Additional exclusion criteria: massive exsanguinating bleeding, an acute coronary syndrome, symptomatic peripheral vasculopathy, stroke, transient ischemic attack, or transfusion within the previous 90 d; a recent history of trauma or surgery; lower gastrointestinal bleeding; a previous decision on the part of the attending physician that the patient should avoid specific medical therapy; and a clinical Rockall score of 0 with a hemoglobin level $> 12$ g/dL

quality, and another two studies were scored as 1 point and considered to be of lower quality (Table 4).

### Death

Three studies reported the incidence of death in two

**Table 3** Baseline characteristics of patients in included studies

Ref.	Groups	No. Patients	Age (yr)	Sex (male: female)	Source of bleeding	Hematocrit at admission	Hemoglobin at admission (g/dL)
Blair <i>et al</i> <sup>[25]</sup>	Restrictive transfusion	26	60 ± 4.5	2:1	Gastric ulcer ( <i>n</i> = 4); duodenal ulcer ( <i>n</i> = 13); carcinoma ( <i>n</i> = 2); Mallory-Weiss tear ( <i>n</i> = 3); not visualized ( <i>n</i> = 4)	29 ± 1.6	NA
	Liberal transfusion	24	64 ± 3.6	2:1	Gastric ulcer ( <i>n</i> = 2); duodenal ulcer ( <i>n</i> = 17); carcinoma ( <i>n</i> = 1); Mallory-Weiss tear ( <i>n</i> = 2); not visualized ( <i>n</i> = 2)	28 ± 1.2	NA
Elizalde <i>et al</i> <sup>[24]</sup>	Restrictive transfusion	8	60 ± 4	5/3	Bleeding from esophageal varices ( <i>n</i> = 7); bleeding from gastric varices in the fundus of the stomach ( <i>n</i> = 1)	27.0 ± 1.3	91.5 ± 6.8
	Liberal transfusion	8	64 ± 2	4/4	Bleeding from esophageal varices ( <i>n</i> = 7); bleeding from gastric varices in the fundus of the stomach ( <i>n</i> = 1)	27.0 ± 1.3	90.5 ± 3.96
Villarejo <i>et al</i> <sup>[23]</sup>	Restrictive transfusion	14	56.8 ± 12.8	9/5	Mallory Weiss ( <i>n</i> = 2); erosive gastritis ( <i>n</i> = 14); erosive gastroduodenitis ( <i>n</i> = 4); Forrest gastric ulcer ( <i>n</i> = 10); Forrest duodenal ulcer ( <i>n</i> = 10); erosive duodenitis ( <i>n</i> = 1)	26.9 ± 4.29	8.76 ± 2.47
	Liberal transfusion	13	45.3 ± 14.6	9/4	Mallory Weiss ( <i>n</i> = 2); erosive gastritis ( <i>n</i> = 14); erosive gastroduodenitis ( <i>n</i> = 4); Forrest gastric ulcer ( <i>n</i> = 10); Forrest duodenal ulcer ( <i>n</i> = 10); erosive duodenitis ( <i>n</i> = 1)	28.3 ± 5.59	8.85 ± 2.53
Villanueva <i>et al</i> <sup>[14]</sup>	Restrictive transfusion	444	NA	NA	Peptic ulcer ( <i>n</i> = 228); gastroesophageal varices ( <i>n</i> = 101); Mallory-Weiss tears ( <i>n</i> = 25); erosive gastritis or esophagitis ( <i>n</i> = 38); neoplasms ( <i>n</i> = 16); other ( <i>n</i> = 36)	NA	9.6 ± 2.2
	Liberal transfusion	445	NA	NA	Peptic ulcer ( <i>n</i> = 209); gastroesophageal varices ( <i>n</i> = 109); Mallory-Weiss tears ( <i>n</i> = 30); erosive gastritis or esophagitis ( <i>n</i> = 29); neoplasms ( <i>n</i> = 20); other ( <i>n</i> = 48)	NA	9.4 ± 2.4

**Table 4** Quality assessment of included studies

Ref.	Randomization	Blinding	Withdrawals and dropouts	Jadad Score
Blair <i>et al</i> <sup>[25]</sup>	Yes (inadequate)	No	Unclear	1
Elizalde <i>et al</i> <sup>[24]</sup>	Yes (inadequate)	Unclear	Unclear	1
Villarejo <i>et al</i> <sup>[23]</sup>	Yes (adequate)	Unclear	Clear	3
Villanueva <i>et al</i> <sup>[14]</sup>	Yes (adequate)	Unclear	Clear	3

groups<sup>[14,23,25]</sup>. One study did not observe any death during the study period in both groups<sup>[23]</sup>, so the OR of this study was not estimable. Another two studies showed a higher incidence of death in patients receiving liberal transfusion<sup>[14,25]</sup>. Heterogeneity among the three studies was not significant ( $I^2 = 0\%$ ;  $P = 0.47$ ). Using a fixed-effect model, the pooled OR was significant (OR = 0.52, 95%CI: 0.31-0.87,  $P = 0.01$ ) (Figure 2A).

### Rebleeding

Two studies reported the incidence of rebleeding in two groups<sup>[14,25]</sup>. Both studies showed a higher incidence of rebleeding in patients receiving liberal transfusion. Heterogeneity among the two studies was significant ( $I^2 = 74\%$ ;  $P = 0.05$ ). Using a random-effect model, the pooled OR was not significant (OR = 0.26, 95%CI: 0.03-2.10,  $P = 0.21$ ) (Figure 2B).

### Length of hospitalization

Two studies reported the length of hospitalization in two groups<sup>[14,23]</sup>. Heterogeneity among the two studies was not significant ( $I^2 = 0\%$ ;  $P = 0.99$ ). Using a fixed-effect mod-

el, the pooled standard mean difference was significant (standard mean difference: -0.17, 95%CI: -0.30--0.04,  $P = 0.009$ ) (Figure 2C).

### Amount of blood transfused

Four studies reported the amount of blood transfused in two groups<sup>[14,23,25]</sup>. Heterogeneity among the four studies was significant ( $I^2 = 54\%$ ;  $P = 0.09$ ). Using a random-effect model, the pooled standard mean difference was significant (standard mean difference: -0.74, 95%CI: -1.15--0.32,  $P = 0.0005$ ) (Figure 2D).

### Hematocrit

Three studies reported the value of hematocrit at discharge or after expansion in two groups<sup>[23,25]</sup>. Heterogeneity among the three studies was significant ( $I^2 = 85\%$ ;  $P = 0.001$ ). Using a random-effect model, the pooled standard mean difference was not significant (standard mean difference: -1.07, 95%CI: -2.36-0.21,  $P = 0.10$ ) (Figure 2E).

### Hemoglobin

Three studies reported the hemoglobin concentration at discharge or after expansion in two groups<sup>[14,23,24]</sup>. Heterogeneity among the three studies was significant ( $I^2 = 56\%$ ;  $P = 0.10$ ). Using a random-effect model, the pooled standard mean difference was significant [standard mean difference: -1.12, 95%CI: -1.73--0.51,  $P = 0.0003$ ] (Figure 2F).

### Sensitivity analysis

Sensitivity analyses were performed after one study with

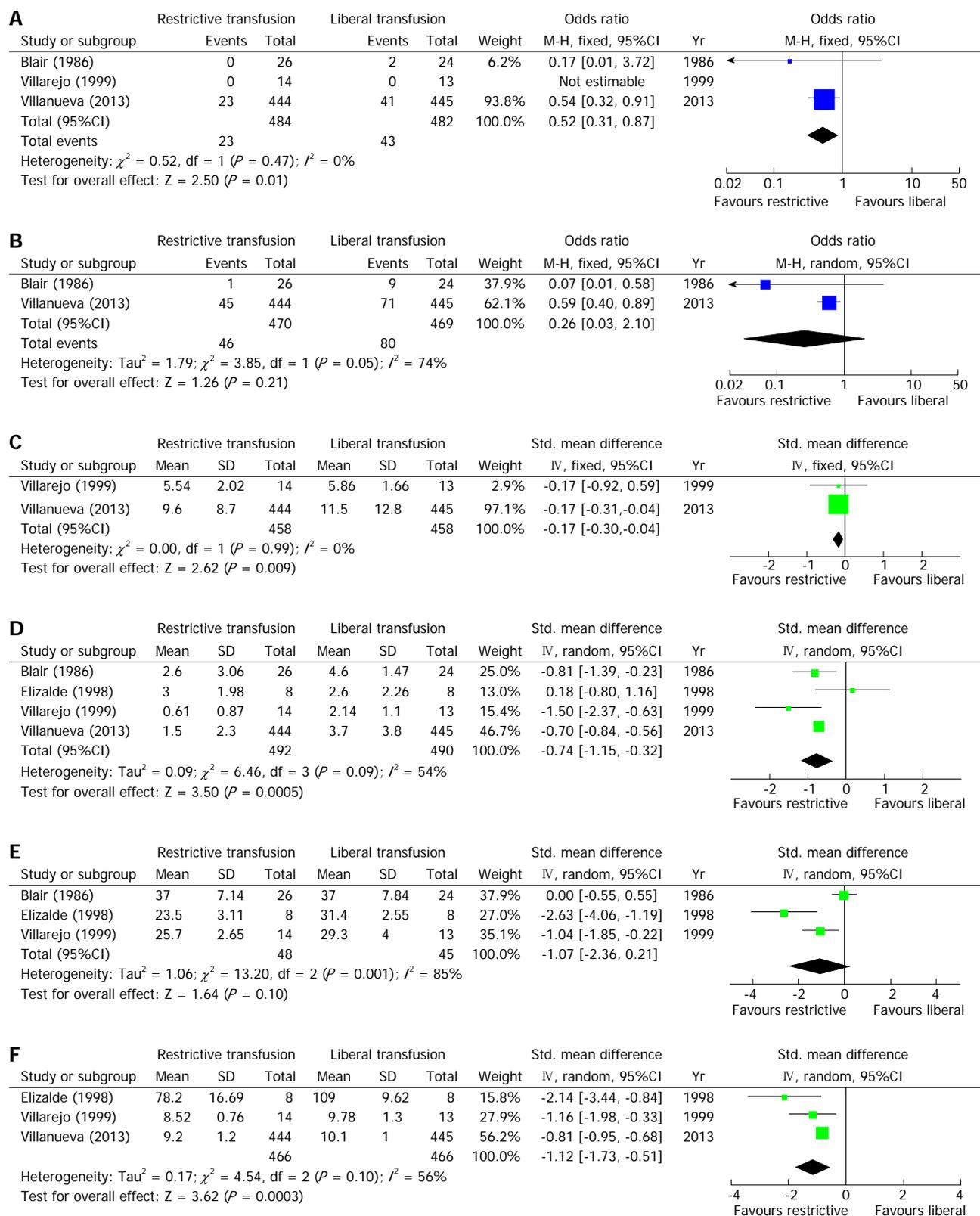


Figure 2 Forest plots comparing the incidence of death (A), incidence of rebleeding (B), length of hospitalization (C), amount of blood transfused (D), and hematocrit (E) and hemoglobin (F) level at discharge or after expansion between patients treated with restrictive and liberal transfusion. Studies are arranged by publication year.

a high proportion of loss to follow-up was excluded<sup>[23]</sup>. Results of all meta-analyses were consistent with those of previous meta-analyses. For death, the pooled OR was 0.52

(95%CI: 0.31-0.87,  $P = 0.01$ ). For length of hospitalization, the pooled standard mean difference was -0.17 (95%CI: -0.31--0.04,  $P = 0.01$ ). For amount of blood transfused,

the pooled standard mean difference was -0.64 (95%CI: -0.97--0.30,  $P = 0.0002$ ). For hematocrit, the pooled standard mean difference was -1.23 (95%CI: -3.79-1.34,  $P = 0.35$ ). For hemoglobin, the pooled standard mean difference was -1.31 (95%CI: -2.57--0.06,  $P = 0.04$ ).

## DISCUSSION

This updated meta-analysis of randomized controlled trials showed the following important findings. First, restrictive transfusion could significantly decrease the incidence of death in patients with upper gastrointestinal bleeding. The survival benefit of restrictive transfusion might be attributed to a lower incidence of further bleeding and transfusion-related adverse events. Second, two randomized controlled trials were included in our meta-analysis and unanimously supported the effectiveness of restrictive transfusion in decreasing the incidence of rebleeding. Our meta-analysis demonstrated a trend in decreasing the incidence of rebleeding in patients treated with restrictive transfusion, but it did not reach any statistical significance. This unexpected finding could be explained by the fact that a random-effect model was employed due to a significant heterogeneity among studies, and the number of patients included in the two randomized controlled trials was substantially different. Third, patients treated with restrictive transfusion had a significantly shorter length of hospitalization and a smaller amount of blood transfused, although the value of hematocrit and hemoglobin at discharge or after expansion was lower in patients treated with restrictive transfusion than in those with liberal transfusion. Generally, these findings accorded with the current international consensus recommendation in which restrictive transfusion should be employed in patients with upper gastrointestinal bleeding. However, we should acknowledge that the threshold of restrictive transfusion strategy was various among these included studies. Accordingly, further studies should be warranted to elucidate the accurate threshold of transfusion in these patients.

The strengths of our study were as follows. First, only randomized controlled trials were included into our meta-analysis. Second, a comprehensive literature search was performed by searching three databases. Third, no publication language was restricted. One Spanish-language full text paper was retrieved by contacting the journal secretary and was translated by Google<sup>[23]</sup>. Fourth, a random-effect model was employed to produce a conservative result with wider confidence intervals, as the heterogeneity among studies was significant<sup>[19]</sup>.

Several limitations of our study should be fully emphasized. First, the publication date of the four studies spanned from 1986 to 2013. This was a relatively long time during which many diagnostic techniques and treatment modalities had been improved. But it should be noted that heterogeneity among studies was not significant for death. Second, three earlier randomized controlled trials had a relatively small sample size<sup>[23-25]</sup>. By comparison, the latest randomized controlled trial had a

larger number of participants included<sup>[14]</sup>. Therefore, the weight of this trial was larger than that of other studies in meta-analyses. In future, more randomized controlled trials are needed to perform a further meta-analysis regarding this topic. Third, one randomized controlled trial had a relatively high proportion of loss to follow-up, thereby increasing the possibility of selection bias<sup>[23]</sup>. Fourth, one randomized controlled trial primarily aimed to observe the hemodynamic effects of an acute increase in blood hemoglobin levels<sup>[24]</sup>. Thus, the data regarding the survival and rebleeding could not be extracted. Fifth, results of one large randomized controlled trial were partially published in two previously published abstracts<sup>[21,22]</sup>. A total of 277 patients with cirrhosis with acute variceal bleeding were enrolled in this study<sup>[14]</sup>. By contrast, the outcome of 147 participants with cirrhosis with acute variceal bleeding was described in one abstract, and the outcome of 214 participants was reported in another abstract<sup>[21,22]</sup>. Importantly, no interim analysis or so called “early stopping rule” was planned in the study. Accordingly, the misbehavior that the authors looked at the data and reported the partial results before the study was completed would introduce the chance of falsely rejecting the null hypothesis (*i.e.*, type I error). Finally, we could not do a meta-analysis according to the different source of bleeding due to a small number of studies included.

In conclusions, this meta-analysis provides preliminary evidence to support that the restrictive transfusion should be employed in patients with upper gastrointestinal bleeding, although the limitation of our study is obvious. Further well designed and conducted randomized controlled trials should be warranted to confirm whether or not restrictive transfusion should be beneficial in different sources of upper gastrointestinal bleeding.

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## COMMENTS

### Background

Acute upper gastrointestinal bleeding is a common cause for emergency hospitalization with a relatively high morbidity and mortality. Red blood cell transfusion is often required in such patients due to the reduction of tissue perfusion after acute blood loss. Current international consensus recommends restrictive transfusion for upper gastrointestinal bleeding. However, this recommendation is largely based on expert opinions.

### Research frontiers

Recently, several large-scale observational studies demonstrated that blood transfusion after nonvariceal upper gastrointestinal bleeding might increase the rate of mortality and rebleeding. More recently, a large and well-organized randomized controlled trial showed a significant benefit of restrictive transfusion strategy in improvement of outcome in patients with upper gastrointestinal bleeding.

### Innovations and breakthroughs

A previous Cochrane Collaboration systematic review of three randomized

controlled trials has shown a tendency in decreasing the mortality of patients with upper gastrointestinal bleeding after restrictive transfusion. But a small number of participants and a high proportion of missing data limit the significance of these findings. These authors concluded that their review might not provide useful data regarding outcomes following red blood cell transfusion for acute upper gastrointestinal bleeding. Thus, authors performed an updated meta-analysis of randomized controlled trials to compare the outcome of upper gastrointestinal bleeding between patients treated with restrictive and liberal transfusion. Results of their meta-analysis demonstrated that patients receiving restrictive transfusion had a lower incidence of death and rebleeding, a shorter length of hospitalization, and a smaller amount of blood transfused than those receiving liberal transfusion.

### Applications

Evidence suggested that restrictive transfusion should be employed in patients with upper gastrointestinal bleeding.

### Peer review

Restrictive transfusion for acute upper gastrointestinal bleeding will become a hot topic in recent years. This study is very interesting. It is valuable to be published.

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**L- Editor** A **E- Editor** Wang CH



## Angiotensin-II inhibitor (olmesartan)-induced collagenous sprue with resolution following discontinuation of drug

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Author contributions: Nielsen JA, Steephen A and Lewin M contributed to the conception, design and acquisition of data, and approved the final version to be published; Steephen A analyzed and interpreted the endoscopy; Lewin M analyzed and interpreted the gastrointestinal pathology; Nielsen JA and Lewin M drafted the article and revised it critically for important intellectual content.

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### Abstract

Collagenous sprue (CS) is a pattern of small-bowel injury characterized histologically by marked villous blunting, intraepithelial lymphocytes, and thickened sub-epithelial collagen table. Clinically, patients present with diarrhea, abdominal pain, malabsorption, and weight loss. Gluten intolerance is the most common cause of villous blunting in the duodenum; however, in a recent case series by the Mayo Clinic, it has been reported that olmesartan can have a similar effect. In this case report, a 62-year-old female with a history of hypothyroidism and hypertension managed for several years with olmesartan presented with abdominal pain, weight loss, and nausea. Despite compliance to a gluten-free diet, the patient's symptoms worsened, losing 20 pounds in 3 wk. Endoscopy showed thickening, scalloping, and mosaiform changes of the duodenal mucosa. The biopsy showed CS characterized by complete villous atrophy, lymphocytosis, and thickened

sub-epithelial collagen table. After 2 mo cessation of olmesartan, the patient's symptoms improved, and follow-up endoscopy was normal with complete villous regeneration. These findings suggest that olmesartan was a contributing factor in the etiology of this patient's CS. Clinicians should be aware of the possibility of drug-induced CS and potential reversibility after discontinuation of medication.

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**Key words:** Collagenous sprue; Celiac disease; Olmesartan; Patient-drug interaction; Duodenum

**Core tip:** Collagenous sprue (CS) is a pattern of small-bowel injury characterized histologically by marked villous blunting, intraepithelial lymphocytes, and thickened sub-epithelial collagen table. Clinically, patients present with diarrhea, abdominal pain, malabsorption, and weight loss, which raises suspicion of celiac disease. Clinicians should be aware of the possibility of drug-induced CS and potential reversibility after discontinuation of medication.

Nielsen JA, Steephen A, Lewin M. Angiotensin-II inhibitor (olmesartan)-induced collagenous sprue with resolution following discontinuation of drug. *World J Gastroenterol* 2013; 19(40): 6928-6930 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6928.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6928>

### INTRODUCTION

Collagenous sprue (CS) is a pattern of small-bowel injury characterized histologically by marked villous blunting, increased intraepithelial lymphocytes, and thickened collagen table. Clinically, patients present with diarrhea, abdominal pain, malabsorption, and subsequent weight

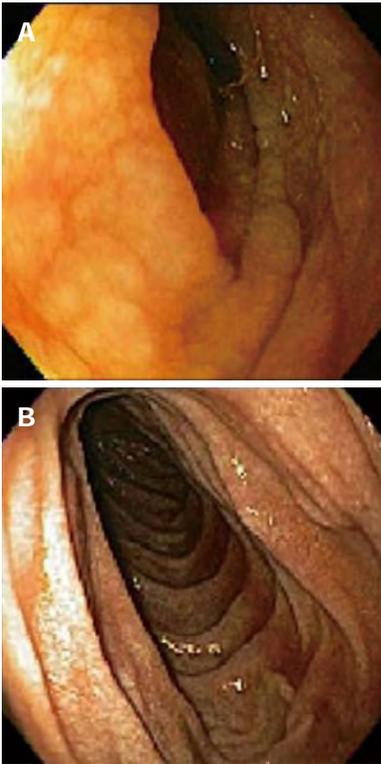


Figure 1 Scalloped mucosa (A) and normal duodenum (B) (endoscopy).

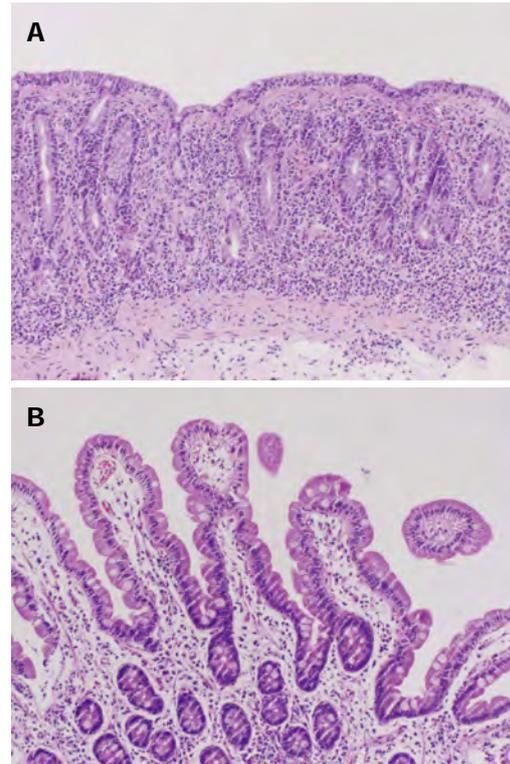


Figure 2 Complete duodenal villous blunting (A) and villous regeneration (B) (hematoxylin and eosin,  $\times 400$ ).

loss. Gluten intolerance is the most common cause of villous blunting in the duodenum; however, in contrast to celiac disease, many patients with CS do not respond to a gluten-free diet<sup>[1]</sup>. Recently, the Mayo Clinic reported in a series of 22 cases that olmesartan can cause a similar severe spruelike enteropathy<sup>[2]</sup>. We report one such case of a woman who had been managing her hypertension with olmesartan for the previous couple years.

## CASE REPORT

A 62-year-old female with a history of hypothyroidism and hypertension presented with abdominal pain, weight loss, change in bowel habits, nausea, and increased bloating/gas; she denied any new medications or nonsteroidal antiinflammatory drugs use. Initial endoscopy was normal; however, the histologic findings showed a CS characterized by complete villous atrophy, up to 100 intraepithelial lymphocytes per 100 epithelial cells, and focally thickened sub-epithelial collagen table. Immunohistochemical stains showed prevalent CD3 positive intraepithelial lymphocytes with no evidence of lymphoma. Celiac markers and anti-enterocyte antibodies were negative; however, histocompatibility leukocyte antigen (HLA)-DQ2 was present. Despite compliance to a gluten-free diet, the patient's symptoms worsened, losing 20 pounds in 3 wk. A second esophagogastroduodenoscopy (EGD) showed thickening and scalloping of duodenal mucosa (Figure 1A). Subsequent histology revealed increased thickness of the collagen band, compared to the previous biopsies, persistent complete villous blunting, and intra-

epithelial lymphocytosis (Figures 2A and 3A). A few days later, the patient was admitted to the Emergency Department for bloody stools and advised to discontinue taking olmesartan because her blood pressure was "normotensive". After cessation of olmesartan the patient's symptoms improved, and 3 mo later EGD (Figure 1B) and biopsy findings were normal, with histologic examination demonstrating complete villous regeneration in the duodenum (Figures 2B and 3B). These findings suggest that olmesartan was a contributing factor in the etiology of this patient's CS.

## DISCUSSION

Many authors still regard CS as a part of the spectrum of celiac disease and designate non-responsive patients as being a "refractory sprue"<sup>[3]</sup>. Both infectious agents and allergic reactions are speculated to be involved in the mucosal injury for a CS, but the etiology and pathogenesis are still unknown<sup>[4]</sup>. Previous accounts of non-gluten sensitivity-related small bowel villous flattening have been reported. In one case series, seven patients all experienced symptoms suggestive of gluten sensitivity and had morphologically-similar mucosal injury in their small bowel biopsy specimens. Regardless of their gluten consumption, all patients experienced clinical improvement and mucosal regeneration. The cause and resolution of their injury is unknown, demonstrating that celiac sprue is not the only disease which can cause villous blunting<sup>[4]</sup>.

In a recent study at Mayo Clinic, 22 patients with

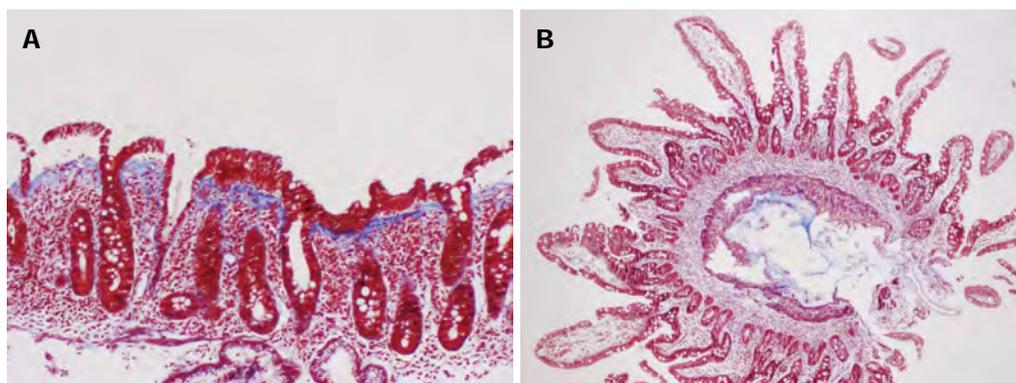


Figure 3 Thickened collagen table (A) and normal histology (B) (trichrome, × 400).

unexplained chronic diarrhea and enteropathy and no response to treatments for celiac disease experienced clinical improvement after suspension of olmesartan. All patients had either partial or total duodenal villous atrophy, 6 of which showed a thickened collagen table. Additionally, 7 patients had collagenous or lymphocytic gastritis, and 5 patients had microscopic colitis. Many of these patients were on olmesartan for months or even years before onset of symptoms. Follow-up biopsy confirmed histologic improvement of the duodenum in 18 patients with sprue-like changes<sup>[2]</sup>, demonstrating that CS can be induced by an autoimmune response to drugs and that morphologic pattern of injury is not necessarily indicative of underlying, specific etiology. As in our patient, 68% of these patients had HLA-DQ2, which is significantly higher than the prevalence reported in the general population of 25%-30%<sup>[2]</sup>.

In conclusion, we report a clear case of an angiotensin- II inhibitor that caused villous blunting of the duo-

denum and gastrointestinal symptoms similar to those of celiac disease. Clinicians should be aware of the possibility of olmesartan-induced CS, with potential reversibility after discontinuation of the drug.

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P- Reviewer **Bian ZX** S- Editor **Zhai HH** L- Editor **A**  
E- Editor **Ma S**



## Resolution of an esophageal leak and posterior gastric wall necrosis with esophageal self-expandable metal stents

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### Abstract

The use of weight reduction surgeries has increased over the years with a higher proportion of these surgeries being sleeve gastrectomies, this has been associated with some complications including staple line leaks. We report a 32-year-old male who had undergone a laparoscopic gastric band surgery and subsequently a laparoscopic sleeve gastrectomy, this was complicated by both an staple line leak at the gastroesophageal junction as well as a large (> 4 cm) posterior gastric wall defect due to gastric wall necrosis. We used two co-axially inserted self-expandable stents (SEMS) in the management of this patient, 5 stents were used over repeated endoscopy sessions and 20 wk. Both defects had resolved without the need for surgical intervention. This is the first reported case where SEMS are used for

both a staple line leak as well as a gastric wall defect. We also review the literature on the use of SEMS in the management of leaks post weight reduction surgeries.

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**Key words:** Stents; Self-expandable metal stents; Laparoscopic sleeve gastrectomy; Staple line leak; Esophageal leak; Gastric necrosis

**Core tip:** Management of staple line leaks has incorporated, in recent years, a non-surgical approach which mainly depends on elimination of oral intake, parenteral nutrition, antimicrobial therapy, and drainage procedures and more recently the use of esophageal self-expandable metal stents for sealing of these leaks as well as the induction of tissue hyperplasia that would close these leaks. This case is the first, to our knowledge, that reports the successful use of self-expandable metal stents for the closure of a posterior gastric wall necrosis as a consequence of repeated bariatric surgery.

Almadi MA, Aljebreen AM, Bamihriz F. Resolution of an esophageal leak and posterior gastric wall necrosis with esophageal self-expandable metal stents. *World J Gastroenterol* 2013; 19(40): 6931-6933 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6931.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6931>

### INTRODUCTION

The morbidity and mortality associated with obesity has been well recognized and in some clinical settings the method to curtail such an epidemic is *via* weight reduction surgeries, such an intervention is associated with some complications of which surgical leaks are one of the most unfavorable as they are associated with considerable morbidity and mortality<sup>[1]</sup>. The management of

these complications has evolved from surgical reoperation to a more conservative approach with elimination of oral intake, parenteral nutrition, antimicrobial therapy, and drainage<sup>[1]</sup>. A more recent trend has been to use esophageal self-expandable metal stents (SEMS) or self-expandable plastic stents (SEPS)<sup>[1]</sup> as a method of occluding these leaks. We present an unusual case where SEMS were used in the management of a patient who developed both a staple line leak at the gastroesophageal junction as well as necrosis of the posterior gastric wall.

## CASE REPORT

A 32-year-old male was referred to our institution with a diagnosis of an esophageal leak after a laparoscopic sleeve gastrectomy (LSG). The patient had already undergone a laparoscopic gastric band weight reduction surgery about 5 years before his presentation with a sub-optimal weight loss reduction. Subsequently the patient underwent a LSG and in the first postoperative week the patient developed clinical signs of an enteric leak with the development of tachycardia as well as fever.

A barium swallow demonstrated a leak at the level of the distal end of the esophagus and a computerized tomography (CT) demonstrated a fluid collection close to the surgical bed. The patient was started on broad-spectrum antibiotics and oral intake was stopped. An endoscopic line of management was planned and an esophagogastroduodenoscopy (EGD) was performed and demonstrated an opening in the distal esophagus that corresponded to the leak seen on a barium study, distal to that defect the pouch above the gastric band was seen and a large defect was seen in the posterior wall of the stomach remnant, which through fluid and the drains left post surgery could be seen (Figures 1 and 2). A single A 12 cm fully-covered SEMS, Niti-S (Taewong Medical, Seoul, South Korea), was deployed with the proximal end of the stent left above the level of the leak and the distal end was just proximal to the pylorus.

A month latter the patient presented with nausea and vomiting and the stent was found to have migrated, a repeat EGD found that the distal end of the SEMS caused an ulcer in the antrum of the stomach, The stent was removed and the patient was kept on a proton pump inhibitor and 12 d later two 15 cm long partially covered SEMS, Ultraflex (Microvasive, Boston Scientific, United States), were deployed in a co-axial fashion, the proximal end of the first stent lied at 30 cm from the incisors above the level of the leak while the distal end of the second stent lied in the duodenum (Figure 3). A few days after the procedure the patient was started on a liquid diet and subsequently the output from the drains had stopped. After 11 and half weeks a rat-tooth forceps was used for removing both stents but the defects in the lower esophagus and the stomach were still present although they had decreased substantially in size, another two partially covered SEMS esophageal stents (Ultraflex) were inserted in a similar fashion to the initial insertion. After



Figure 1 Appearance of a large defect in the posterior wall of the stomach with the post-operative drains seen through it.



Figure 2 A closer look at the drains through the defect.

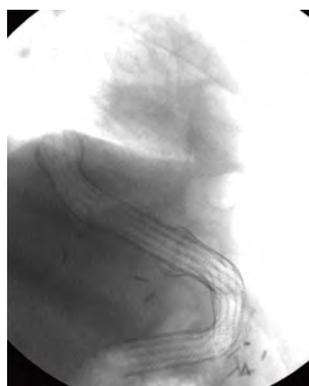


Figure 3 Two co-axially inserted partially covered self-expandable esophageal metal stents extending from the esophagus to the duodenum.

4 wk both stents were removed easily (Figure 4) and the defects had resolved. The patient had a subsequent CT scan of the abdomen with oral contrast that demonstrated resolution of both leaks and the drains were removed.

## DISCUSSION

The use of enteric stents has evolved from the management of malignant diseases to benign strictures as well as leaks. LSGs are complicated by leaks in 2.2%<sup>[3]</sup>-2.4%<sup>[4]</sup>



Figure 4 Self expandable metal stent being removed from the distal aspect with a rat-tooth forceps.

of cases based on two systematic reviews with the majority of these leaks (85%-89%) being located in the proximal third of the stomach near the gastroesophageal junction<sup>[1,4]</sup> and are associated with a non-trivial mortality rate (6%-14.7%)<sup>[1]</sup>. The management options for this complication vary from repeat surgery to a more conservative management with an attempt to seal these leaks through enteric stenting. Surgical management has been associated with a high morbidity (up to 50%) and mortality (2%-10%)<sup>[1]</sup>, as a result, initial management has moved towards a more conservative endoscopic treatment. Although there are no randomized controlled trials to assess the efficacy of this approach, a systematic review that included 25 studies, incorporating 267 patients showed that the success rate with the use of SEMS was about 85% with no difference in the rate of clinical success between the type of SEMS used whether they were partially or fully-covered SEMS or SEPS ( $P = 0.97$ )<sup>[5]</sup>, of note this systematic review included cases who had esophageal anastomotic leaks or a benign rupture of the esophagus<sup>[5]</sup>. A second meta-analysis for patients who exclusively had leaks post bariatric surgery and were managed by stenting found a success rate of 87.8% (95%CI: 79.4%-94.2%)<sup>[6]</sup>.

The case we present is unique due to the simultaneous presence of a leak at the gastroesophageal junction as well as a large area of necrosis in the posterior wall of the stomach both of which were eliminated using SEMS for a prolonged period of time and the use of a total of 5 stents and five endoscopy sessions spanning a period of 20 wk. SEMS are usually kept in place on average for 6 wk<sup>[5]</sup>. In the case reported the duration of stenting was 20 wk mainly due to the large defect seen in the gastric wall.

Stent migration occurred with the first fully covered

SEMS that necessitated its removal and exchange. This complication is acceptable as the rate of migration in general in a meta-analysis was 16.9% (95%CI: 9.3%-26.3%)<sup>[6]</sup> and van Boeckel *et al*<sup>[5]</sup> found that fully covered SEMS had a higher migration rate when compared to partially covered SEMS (26% *vs* 12% respectively,  $P \leq 0.001$ )<sup>[5]</sup>. Although endoscopic repositioning/removal might suffice in the majority of cases, as in our case, some of these stents passed per-rectum spontaneously or had to be removed surgically<sup>[6]</sup>.

The longer the SEMS are left in place the higher the risk of ingrowth of tissue within the stent and subsequently the more difficult its removal<sup>[1]</sup>. Removal of these SEMS usually require the insertion of a SEPS within the SEMS to induce ingrowth tissue necrosis and after a few days both the SEMS and SEPS can be removed, successful removal of the SEMS after leak closure is 91.6% (95%CI: 84.2%-96.8%)<sup>[6]</sup>. We intentionally removed the SEMS after predefined durations to reduce the risk of failure to remove the SEMS. Furthermore, due to the initial use of fully covered and then partially covered SEMS they were removed without requiring the insertion of SEPS.

This case is the first to our knowledge where both a staple line leak post LSG and posterior gastric wall necrosis were simultaneously treated using SEMS without the need for repeated surgery.

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**E-Editor** Zhang DN



## Adjuvant surgery for advanced extrahepatic cholangiocarcinoma

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### Abstract

Patients with Stage IV cholangiocarcinoma are currently not considered to be surgical candidates and are typically offered systemic chemotherapy. Recently, several novel systemic chemotherapy regimens have allowed an initially unresectable cholangiocarcinoma to be resectable. The aim of this article is to present the usefulness of adjuvant surgery in a case of advanced cholangiocarcinoma that was successfully treated with gemcitabine. A 72-year-old man was diagnosed with distal cholangiocarcinoma with liver metastases (cT2N0M1, Stage IV). He underwent metal stent placement in the duodenum to alleviate jaundice. After 18 courses of chemotherapy using gemcitabine without severe drug toxicities, a computed tomography scan showed that the liver metastases in S6 and S7 had disappeared. The patient underwent subtotal stomach-preserving pancreaticoduodenectomy and lymph node dissection. The pathological stage was pT2N0M0, Stage I B. The patient underwent 6 cycles of adjuvant chemotherapy using gemcitabine. The patient is alive

and well 6 years and 9 mo after the diagnosis.

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**Key words:** Extrahepatic cholangiocarcinoma; Adjuvant surgery; Conversion surgery; Chemotherapy; Gemcitabine

**Core tip:** Patients with Stage IV cholangiocarcinoma are currently not considered to be surgical candidates and are typically offered systemic chemotherapy. Recently, several novel systemic chemotherapy regimens have allowed an initially unresectable cholangiocarcinoma to be resectable. In a patient with advanced extrahepatic cholangiocarcinoma, gemcitabine (GEM) induced a dramatic reduction of the tumor, which led to curative resection and a long-term survival of 6 years and 9 mo. This result suggests the possibility of advantages of using GEM for the treatment of advanced cholangiocarcinoma, and GEM-based chemotherapy could be performed more often for unresectable cholangiocarcinomas.

Oshiro Y, Takahashi K, Sasaki R, Kondo T, Sakashita S, Ohkohchi N. Adjuvant surgery for advanced extrahepatic cholangiocarcinoma. *World J Gastroenterol* 2013; 19(40): 6934-6938  
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6934.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6934>

### INTRODUCTION

Cholangiocarcinoma continues to exhibit poor survival rates compared with other gastrointestinal malignancies<sup>[1-4]</sup>. Most cholangiocarcinoma patients are not surgical candidates. Patients with Stage IV cholangiocarcinoma are currently inoperable and are typically offered systemic chemotherapy. The most promising approaches involve

the use of single agents such as gemcitabine (GEM), which has been shown to be effective against cholangiocarcinoma in phase II trials<sup>[5-7]</sup>. In these trials, the response rates for GEM ranged from 8% to 36%, and the overall survival (OS) ranged from 6.3 to 16 mo. We describe a rare case of stage IV cholangiocarcinoma with liver metastases that was initially deemed unresectable and became resectable after GEM chemotherapy and showed a favorable outcome.

## CASE REPORT

The patient was a 72-year-old man referred from a local hospital complaining of jaundice. The laboratory data on admission showed the following elevated values: total bilirubin (T-bil), 6.2 mg/dL (normal range, 0.2-1.2 mg/dL); lactic acid dehydrogenase, 243 U/L (124-232 U/L); alkaline phosphatase 354 U/L (120-320 U/L); and  $\gamma$ -glutamyl transpeptidase, 181 U/L (5-55 U/L). All the tumor markers tested were within the normal limits: carcinoembryonic antigen (CEA), 2.3 ng/mL (normal range, < 5.0 ng/mL), and carbohydrate antigen 19-9, 12.0 U/mL (< 37 U/mL). Abdominal computed tomography (CT) and ultrasonography showed mild dilatation of the common bile duct and bilateral dilation of the intrahepatic bile ducts. Abdominal computed tomography angiography (CTA) detected wall thickening in the distal common bile duct, and the lesion was enhanced by contrast (Figure 1). CT and CTA showed two liver metastases, which measured 8 mm (S6) and 8 mm (S7) in diameter (Figure 2). According to the Union Internationale Contre le Cancer (UICC) guidelines, the patient was diagnosed with lower cholangiocarcinoma (cT2N0M1, Stage IV)<sup>[8]</sup>.

The patient underwent successful placement of a self-expandable metal duodenal stent to relieve jaundice. The patient received a total of 18 cycles of GEM. GEM was administered intravenously at a dose of 800 mg/m<sup>2</sup> per day on days 1, 8, and 15 in a 28-d cycle. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria Grading System (Version 2.0, April 1999)<sup>[9]</sup>. Severe drug toxicities (grade 3 or 4) were not observed.

After 18 cycles of chemotherapy, CT showed that the two liver metastases in S6 and S7 disappeared. The tumor was clinically downstaged to Stage I B (cT2N0M0).

Four weeks after the completion of chemotherapy, the operation was successfully performed. Peritoneal lavage cytology demonstrated no cancer cells in the abdominal cavity. No microscopic invasion of the resected bile duct stump was observed in an intraoperative frozen specimen. The patient underwent curative resection consisting of SSPPD with D2 lymphadenectomy without resecting any other organs.

Tumor cells were detected in the distal bile duct upon microscopic examination (Figure 3). According to the UICC guidelines, the pathological classification of the tumor was cT2N0M0 Stage I B. The patient was discharged on postoperative day 61 in good condition.



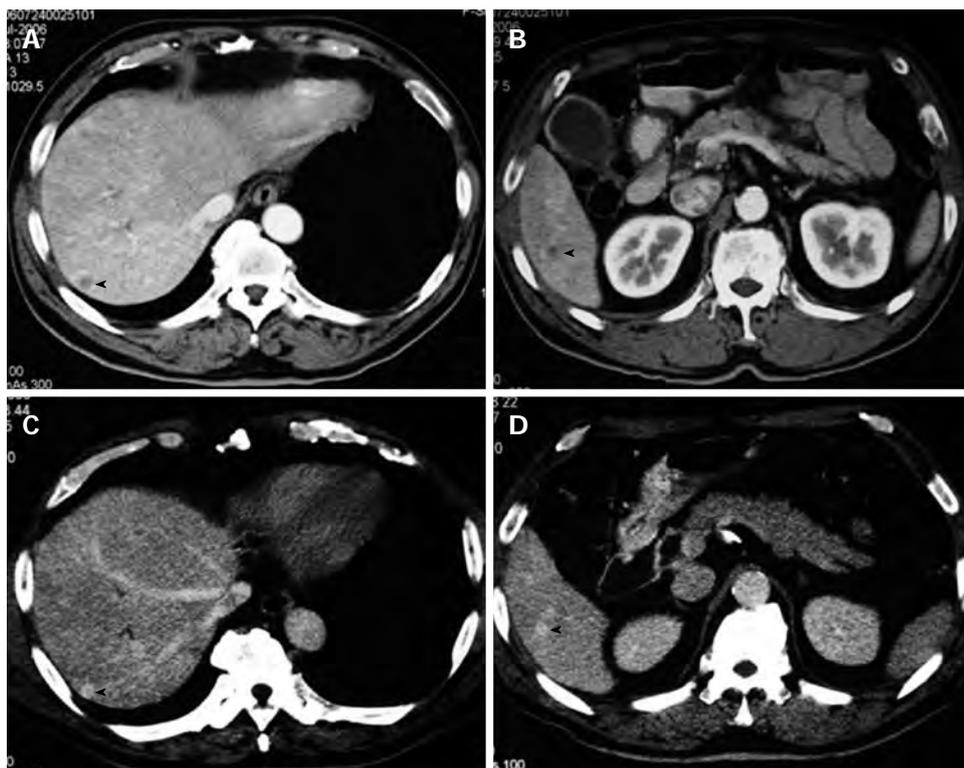
**Figure 1** Abdominal computed tomography angiography image of the common bile duct. Thickening of the wall of the distal common bile duct, which enhances with contrast, was observed (arrowhead).

The patient subsequently received six cycles of adjuvant GEM chemotherapy similar to the preoperative regimen. The patient is alive at 6 years and 9 mo after the diagnosis and 5 years after the surgery.

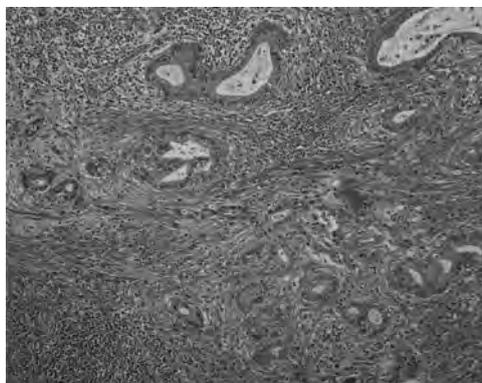
## DISCUSSION

The prognosis of patients with cholangiocarcinoma is poor, with a five-year survival rate of approximately 25% to 55%<sup>[1-4]</sup>. To overcome this clinical challenge, several strategies, including adjuvant chemotherapy, adjuvant radiotherapy, and adjuvant chemoradiotherapy have been considered for treating cholangiocarcinoma<sup>[10-13]</sup>. Few randomized clinical trials have evaluated the utility of adjuvant therapy following R0 resection of cholangiocarcinoma, and most of the current studies are small and retrospective. Therefore, no standard adjuvant modalities have been universally adopted for the treatment of cholangiocarcinoma, and the role of chemotherapy for unresectable cholangiocarcinoma has not been established. Although there has been no standard chemotherapy for cholangiocarcinoma, GEM has been the most actively used agent against cholangiocarcinoma. We treated a patient with advanced extrahepatic cholangiocarcinoma with liver metastases. The patient showed a dramatic response to GEM, which led to curative resection and long-term survival of more than 6 years. GEM may be an effective chemotherapeutic agent for treating cholangiocarcinoma, and a randomized clinical trial needs to be performed.

The feasibility of adjuvant surgery for cholangiocarcinoma has not been determined. Recently, in colorectal, gastric, and pancreatic cancer, several authors have reported “conversion surgery” or “adjuvant surgery”<sup>[14-16]</sup>. Suzuki *et al.*<sup>[14]</sup> demonstrated that adjuvant surgery was effective in 20 advanced gastric cancer patients (Stage IV) based on liver or distant lymph node metastasis. The overall survival of patients in the partial response and curative resection groups was prolonged. The survival of patients with H or N factor was also prolonged when they received curative surgery. However, the survival of



**Figure 2** Abdominal computed tomography and computed tomography angiography images at two different levels are shown. A: Computed tomography (CT) showed a low-density mass measuring 8 mm in diameter located in segment 7 of the liver (arrowhead); B: CT showed a low-density mass measuring 8 mm in diameter in segment 6 (arrowhead); C: Computed tomography angiography (CTA) demonstrated an enhancing mass lesion at the same location as in Figure 2A (arrowhead); D: CTA demonstrated an enhancing mass lesion at the same location as in Figure 2B (arrowhead).



**Figure 3** Histopathologic appearance of the cholangiocarcinoma (hematoxylin and eosin,  $\times 200$ ). Tumor cells were detected in the distal bile duct on microscopic examination.

patients with P factor was not prolonged. Locally advanced pancreatic cancer may be a good indication for adjuvant surgery after sustained favorable responses to chemotherapy, even in patients with initially unresectable disease<sup>[6]</sup>. In 2013, Kato *et al*<sup>[17]</sup> reported that eight patients with initially unresectable advanced biliary tract cancer who underwent adjuvant surgery had significantly longer survival than 14 patients who were unable to undergo surgery. Of the eight patients in the surgery group, four patients had gallbladder carcinoma and four patients had intrahepatic cholangiocarcinoma. To our knowledge, from 1983 to 2013, in the field of bile duct cancer, only

16 cases in nine reports underwent adjuvant surgery, including the cases in the report<sup>[17-25]</sup> (Table 1). Of the 16 patients, 10 patients received GEM, 3 received S-1, 2 received GEM and S-1, 1 received GEM combined with cisplatin and fluorouracil, and 1 received cisplatin/interferon  $\alpha$ -2b/doxorubicin/fluorouracil-combination chemotherapy. None of the 16 cases involved extrahepatic cholangiocarcinoma. To the best of our knowledge, this is the first report of adjuvant surgery for extrahepatic cholangiocarcinoma.

Medical oncologists and surgeons have identified surgical candidates among patients with initially unresectable colorectal and gastric cancer who responded favorably to multimodal treatment<sup>[14,15]</sup>. In some cases, the addition of surgery resulted in increased long-term survival. Surgical resection coupled with multimodal treatment is called “adjuvant surgery.” Surgical resection can be classified as curative (no evidence of remaining disease after surgery) or palliative (remaining disease after surgery). Therefore, adjuvant surgery aims to be curative and not palliative after the response to chemotherapy<sup>[14]</sup>.

In a strategy involving adjuvant surgery, adjuvant chemotherapy is considered necessary after the operation. In our patient, the liver metastases showed a surprising complete response without severe toxicity after GEM chemotherapy. Additionally, the patient received adjuvant chemotherapy using GEM as an outpatient and developed no adverse reactions. In previous phase II studies using single-agent GEM, major adverse reactions included

**Table 1** Cases of advanced bile duct cancer treated with adjuvant surgery following effective chemotherapy

Ref.	Diagnosis	Metastasis/invasion	Chemotherapy regimen	Response
Slupski <i>et al</i> <sup>[18]</sup>	IntraHepatic cholangiocarcinoma	Lung metastases	CDDP, 5-FU, IFN, doxorubicin	PR
Shirabe <i>et al</i> <sup>[19]</sup>	Gallbladder cancer	Para-aortic LNs	GEM, CDDP, 5-FU	PR
Kitajima <i>et al</i> <sup>[20]</sup>	Gallbladder cancer	Dissemination	S-1	CR
Morimoto <i>et al</i> <sup>[21]</sup>	Gallbladder cancer	Liver metastasis	GEM	CR
Kanaji <i>et al</i> <sup>[22]</sup>	IntraHepatic cholangiocarcinoma	Dissemination	S-1	CR
Kim <i>et al</i> <sup>[23]</sup>	IntraHepatic cholangiocarcinoma	Portal vein invasion	GEM	PR
Ohno <i>et al</i> <sup>[24]</sup>	Ampulla of Vater cancer	Liver metastasis	GEM, S-1	CR
Hasegawa <i>et al</i> <sup>[25]</sup>	Gallbladder cancer	Hepatic invasion	S-1, para-aortic LN	PR
Kato <i>et al</i> <sup>[17]</sup>	Intrahepatic cholangiocarcinoma	Hepatic vein invasion	GEM	SD
	Intrahepatic cholangiocarcinoma	Hepatic vein invasion	GEM	PR
	Intrahepatic cholangiocarcinoma	Arterial invasion	GEM	SD
	Intrahepatic cholangiocarcinoma	Insufficient remnant liver volume	GEM	PR
	Gallbladder cancer	Arterial invasion	GEM	SD
	Gallbladder cancer	Arterial invasion	GEM	PR
	Gallbladder cancer	Arterial invasion portal vein invasion	GEM	SD
	Gallbladder cancer	Arterial invasion	GEM	SD

CDDP: Cisplatin; 5-FU: Fluorouracil; IFN: Interferon; PR: Partial response; CR: Complete response; GEM: Gemcitabine; LN: Lymph node; SD: Stable disease.

neutropenia, leukopenia, and anemia were observed with little severe toxicity<sup>[5-7]</sup>. The results suggest that GEM is suitable for outpatients because of its mild toxicity.

The UK ABC-02 study defined the standard of care for unresectable advanced biliary tract cancer<sup>[26]</sup>. Valle *et al*<sup>[26]</sup> reported that cisplatin with GEM (GEMC) was associated with a significant survival advantage compared with GEM alone. The median OS was 11.7 mo for GEMC and 8.1 mo for GEM alone<sup>[26]</sup>. A Japanese trial of 83 patients using the same treatment regimens as UK ABC-02 showed the median survival and overall response rate of GEMC *vs* GEM alone were 11.2 mo *vs* 7.7 mo and 19.5% *vs* 11.9%, respectively. These results were consistent with the results of the UK ABC-02 study. GEMC was found to be effective and well tolerated, which indicates that it could also be a standard regimen for Japanese patients<sup>[27]</sup>.

In conclusion, in a patient with advanced extrahepatic cholangiocarcinoma, GEM induced a dramatic reduction of the tumor, which led to curative resection. The patient was still living 6 years and 9 mo after the study. The results suggest possible advantages of using GEM for the treatment of advanced cholangiocarcinoma. GEM-based chemotherapy could be more commonly administered for unresectable cholangiocarcinoma. Furthermore, "adjuvant surgery" (*i.e.*, R0 resection) may significantly contribute to curing cholangiocarcinoma. An evidence-based consensus should be developed on potentially resectable cholangiocarcinoma with liver metastases in each hospital.

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## Isolated gastric variceal bleeding caused by splenic lymphoma-associated splenic vein occlusion

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### Abstract

Isolated gastric varices (IGV) can occur in patients with left-sided portal hypertension resulting from splenic vein occlusion caused by thrombosis or stenosis. In left-sided portal hypertension, blood flows retrogradely through the short and posterior gastric veins and the gastroepiploic veins, leading to the formation of an IGV. The most common causes of splenic vein occlusion are pancreatic diseases, such as pancreatic cancer, pancreatitis, or a pseudocyst. However, various other cancers, such as colon, gastric, or renal cancers, have also been known to cause splenic vein occlusion. Our patient presented with a rare case of IGV bleeding induced by splenic lymphoma-associated splenic vein occlusion. Splenectomy, splenic artery embolization, and stenting of the splenic vein are the current treatment choices. Chemotherapy, however, is an alternative ef-

fective treatment for splenic vein occlusion caused by chemotherapy-sensitive tumors. Our patient responded well to chemotherapy with a cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisolone regimen, and the splenic vein occlusion resolved after the lymphoma regressed.

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**Key words:** Isolated gastric varices; Splenic vein; Lymphoma; Occlusion; Hematemesis

**Core tip:** Isolated gastric varices occur in patients with splenic vein occlusion caused by thrombosis, stenosis, or cancer, such as pancreatic, colon, gastric, or renal cancers. Here, we describe a rare case of an isolated gastric varices in an elderly woman who presented with tarry stool and bloody vomitus. The diagnostic tests revealed a splenic B-cell lymphoma, which caused splenic vein occlusion that resulted in isolated gastric variceal bleeding. Splenectomy, splenic artery embolization, and stenting of the splenic vein are the current treatment choices. In this patient, chemotherapy was an alternative treatment, and the splenic vein occlusion resolved after the lymphoma regressed.

Chen BC, Wang HH, Lin YC, Shih YL, Chang WK, Hsieh TY. Isolated gastric variceal bleeding caused by splenic lymphoma-associated splenic vein occlusion. *World J Gastroenterol* 2013; 19(40): 6939-6942 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i40/6939.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i40.6939>

### INTRODUCTION

Hematemesis is a medical emergency, and in most cases, it is caused by esophageal varices or peptic ulcers. However, there are other rare, but important and treatable causes of hematemesis, such as isolated gastric vari-

ces. We report one such case and review the literature, focusing on the unusual entities presenting as isolated gastric variceal bleeding.

## CASE REPORT

A 77-year-old woman was admitted to our hospital due to two episodes of bloody vomitus and three episodes of tarry stool. She did not have a significant medical or surgical history or any known allergies, nor was she taking any medication. The physical examination revealed pale conjunctiva without any jaundice, lymphadenopathy, or pedal edema. An abdominal palpation demonstrated an enlarged spleen, palpable below the left costal margin, and a digital rectal examination showed melena. The remainder of the patient's systemic examination results was unremarkable. The blood pressure was 97/55 mmHg, the pulse rate was 96/min, the hemoglobin level was 8.9 g/dL, the platelet count was 118000/ $\mu$ L, and the stool occult blood test was positive. The patient's biochemistry tests, including those for liver and renal function, were within the normal limits, apart from a high serum lactate dehydrogenase level (1112 U/L). Esophagogastroduodenoscopy demonstrated an isolated gastric varices (IGV) at the cardia and high body of the stomach, with active bleeding (Figure 1); thus, sclerotherapy with cyanoacrylate was successfully performed. Abdominal computed tomography (CT) showed a large mass in the enlarged spleen, with near total occlusion of the splenic vein (Figure 2A). This splenic vein occlusion led to the development of a gastric varices at the fundus (Figure 2B), and the portal and superior mesenteric veins were patent. The laboratory findings indicated the patient's carcinoembryonic antigen and carbohydrate antigen 19-9 levels were normal.

The patient underwent ultrasound-guided aspiration biopsy, and histopathology confirmed a high-grade B-cell lymphoma (Figure 3). Thereafter, the patient received chemotherapy with a cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisolone (CHOP) regimen, and the splenic vein occlusion resolved after the lymphoma regressed (Figure 2C).

## DISCUSSION

IGVs are observed in up to 5% of patients with cirrhosis and in up to 10% of patients with non-cirrhotic portal hypertension<sup>[1]</sup>. Such varices can occur in patients with segmental or left-sided portal hypertension (LSPH), and the incidence has increased over the past three decades because of increased awareness of the entity and advances in diagnostic approaches<sup>[2,3]</sup>. When a segment of the portal venous bed is obstructed, varices can develop in the area to decompress the blocked segment. In cases of segmental portal hypertension, the blood drains in a retrograde manner through the short and posterior gastric veins and the gastroepiploic veins. This process results in blood flow and a pressure increase, which causes dilation

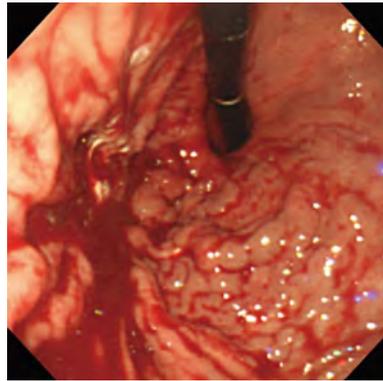
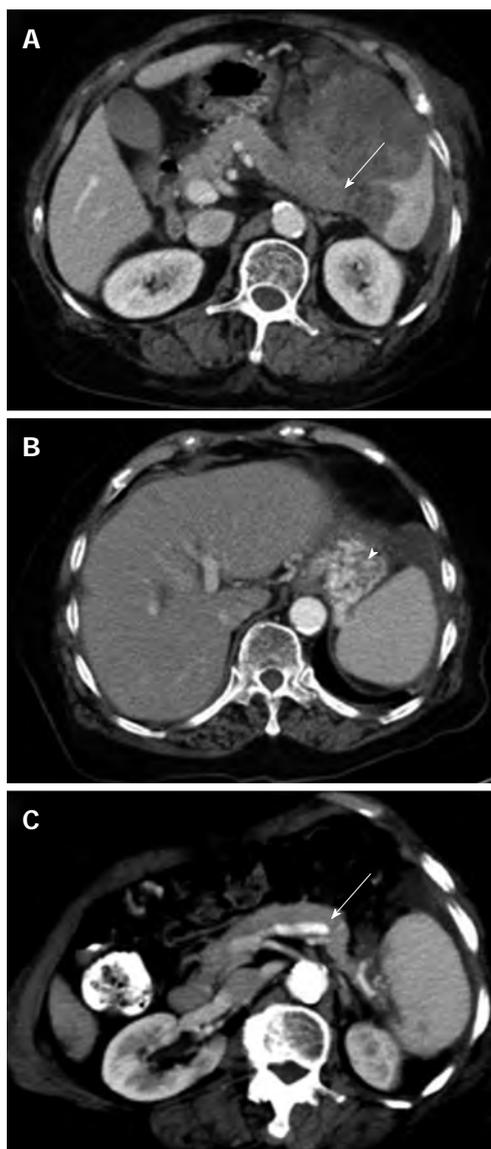


Figure 1 Engorged varices at the cardia and high body of the stomach, with active bleeding.

of the submucosal structures, leading to the formation of a gastric varice<sup>[4]</sup>.

LSPH primarily occurs as a result of splenic vein obstruction, most commonly resulting from pancreatic disorders<sup>[5]</sup>. Because the splenic vein is posterior to the pancreas and in direct contact with it, any type of pancreatic disease, including cancer, pancreatitis, abscess formation, or a pseudocyst, can affect the splenic vein<sup>[6-8]</sup>. In a study by Moosa *et al.*, pancreatitis, diagnosed by either biopsy or surgery, was part of the LSPH etiology in 87 (60%) of 144 cases, while pancreatic malignancy was detected in only 13 (9%) patients<sup>[9,10]</sup>. Various disorders other than pancreatic diseases, such as partial gastrectomy, metastatic carcinoma, retroperitoneal fibrosis, or protein S deficiency, have a wide spectrum of mechanisms involved in causing splenic vein occlusion<sup>[5,11-13]</sup>. Among the metastatic carcinomas, oat cell carcinoma<sup>[14]</sup>, colon cancer<sup>[5]</sup>, gastric cancer<sup>[5]</sup>, renal cancer<sup>[12]</sup>, and rare retroperitoneal liposarcoma<sup>[9]</sup> have been reported to cause splenic vein occlusion. Primary pancreatic lymphoma, with splenic vein thrombosis and IGV bleeding, has been reported as well<sup>[15]</sup>. Our patient had a rare case of splenic B-cell lymphoma that caused splenic vein occlusion and IGV bleeding.

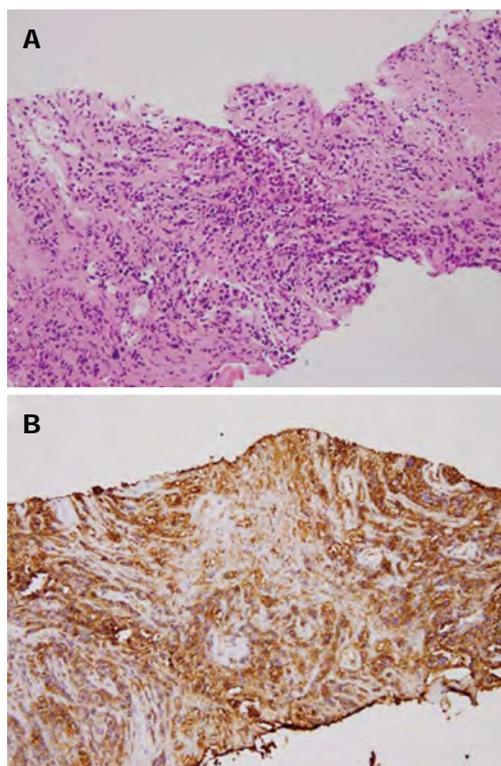
In general, IGVs are asymptomatic and identified incidentally. In symptomatic cases, the most common clinical manifestation is gastrointestinal bleeding from ruptured gastric varices; statistically, 45%-72% of patients with LSPH present with the above-mentioned symptom<sup>[3]</sup>. Splenomegaly and abdominal pain are observed in 71% and 25%-38% of patients with LSPH, respectively<sup>[15,9]</sup>, whereas ascites seldom develop<sup>[16]</sup>. Various radiological examinations can assist in making an IGV diagnosis, including upper gastrointestinal endoscopy, angiography, ultrasonography, contrast-enhanced CT portography, and magnetic resonance imaging. In one study, gastric varices could be accurately diagnosed and localized in nearly 90% of cases by endoscopy<sup>[17]</sup>. Endoscopic ultrasound (EUS) appears to be a more accurate test than transabdominal ultrasonography, which is useful for evaluating the pancreatic parenchyma and splenic vasculature<sup>[18]</sup>. Contrast-enhanced CT portography can



**Figure 2 Imaging findings.** A: Axial contrast-enhanced abdominal computed tomography (CT) at the level of the splenic hilum shows a large low-attenuation mass in the enlarged spleen. The mass involves the splenic vein with near total occlusion (arrow); B: The development of a gastric varice (arrowhead) at the fundus; C: The 6-mo follow-up CT shows that the splenic mass has almost completely resolved, allowing the splenic vein (arrow) to return to patency.

quickly elucidate the portal venous system, but it requires a large amount of iodinated contrast material. Magnetic resonance angiography with gadopentetate dimeglumine is a promising noninvasive test that is being increasingly used to diagnosis patency or thrombosis of the portal venous system<sup>[19]</sup>.

The treatment of IGV bleeding should be directed toward the splenic side of the portal circulation because the pressure increases only on that side<sup>[14]</sup>. Several choices such as conservative therapy (*e.g.*, pharmacotherapy, balloon tamponade, endoscopic therapy with sclerotherapy or band ligation) and interventional radiologic techniques, are available for bleeding control. Japanese endoscopists have reported using a combination technique of endoscopic variceal ligation injection sclerotherapy to treat



**Figure 3 Pathologic findings (hematoxylin/eosin staining) and immunohistochemical staining.** A: The predominant fibrous tissue with necrotic foci and scattered atypical cells characterized by pleomorphic and hyperchromatic nuclei with discohesive arrangement. B: The B-lymphocyte marker (CD20) was strongly positive in the atypical cells.

acute gastric varice bleeding; when using this procedure, the rebleeding rate was low (0%-8%), the gastric varice eradication rate was 85% at up to 2 years post-treatment, and hemostasis was achieved in 100% of patients<sup>[20]</sup>. Transcatheter splenic artery embolization has also been attempted with varying degrees of success, but it has not become a preferred approach. If a patient with active bleeding is unresponsive to conservative management, then splenectomy is indicated. A large splenectomy series that included patients with isolated splenic vein thrombosis showed that none of the patients had recurrent bleeding during the 11-mo mean follow-up period after splenectomy<sup>[9]</sup>.

The current case was a rare incidence of IGV bleeding induced by splenic lymphoma-associated splenic vein occlusion. In this patient, chemotherapy was an alternative treatment for splenic vein occlusion caused by chemotherapy-sensitive tumors. Our patient responded well to chemotherapy, and the splenic vein occlusion resolved after the lymphoma regressed.

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## Laparoscopic treatment of an upper gastrointestinal obstruction due to Bouveret's syndrome

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### Abstract

Bouveret's syndrome is an extremely rare type of gallstone-induced ileus with atypical clinical manifestations, such as abdominal distension and pain, nausea and vomiting, fever or even gastrointestinal bleeding, which may easily be misdiagnosed. In the present case, a 55-year-old male was admitted to the hospital with upper gastrointestinal obstructive symptoms but without pain, fever, jaundice or melena. At first, gastrolithiasis and peptic ulcer combined with pyloric obstruction were suspected after gastroscopy revealed a large, hard stone in the duodenal bulb. A revised diagnosis of Bouveret's syndrome was made following abdominal computed tomography. Subsequently, the patient exhibited a good postoperative recovery after laparoscopic duodenotomy for gallstone removal and subtotal cholecystectomy. The condition of the patient

remained stable after being followed up for 6 mo. The successful application of laparoscopic therapy to treat Bouveret's syndrome has seldom been reported. Laparoscopic enterolithotomy is safe and effective, with good patient tolerability, rapid postoperative recovery and few wound-related complications. The laparoscopic treatment of Bouveret's syndrome is worth exploring.

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**Key words:** Bouveret's syndrome; Gallstone; Gastric outlet obstruction; Laparoscopic therapy; Cholecysto-enteric fistula

**Core tip:** Bouveret's syndrome is a rare cause of gastric outflow obstruction and is easily misdiagnosed. In this case, the patient was middle-aged and did not present any chronic systemic disease. The clinical symptoms were atypical, with neither infection nor abdominal pain. The onset suggested peptic ulcer and pyloric obstruction, which was initially suspected as gastrolithiasis. An imaging study from a year prior provided important data regarding the evolution and diagnosis of the disease. The successful application of laparoscopic therapy to treat Bouveret's syndrome has seldom been reported. This patient obtained satisfactory results after laparoscopic duodenotomy for gallstone removal and subtotal cholecystectomy.

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### INTRODUCTION

Bouveret's syndrome is a rare complication of choleli-

thiasis that was first reported and named by Bouveret in 1896; this condition comprises a series of syndromes caused by a gastric outflow obstruction due to the migration of gallstones to the pylorus and duodenum *via* a gallbladder-duodenal fistula<sup>[1]</sup>. We have only rarely encountered this condition in clinical work, and few cases have been reported in the literature. The most common site of gallstone-induced ileus is the terminal ileum, followed by the proximal ileum and jejunum; only 1%-3% of cases occur in the duodenum, and an obstruction caused by incarcerated stones in the pylorus and duodenal bulb is the rarest form encountered in the clinical setting<sup>[2]</sup>. Gallstone-induced ileus is 3 to 16 times more common in females than in males, and Bouveret's syndrome predominately affects older women<sup>[3]</sup>. Because it often presents in the elderly and with multiple comorbidities, it is associated with a high rate of mortality. The first treatment choice for this syndrome remains debatable.

## CASE REPORT

A 55-year-old male was admitted to the hospital with a 2-wk history of upper abdominal distension and vomiting. Nausea and vomiting frequently occurred 4-5 h after a meal. Undigested food was sometimes present in the vomitus. Pain, fever, jaundice and melena were not reported by the patient. Several assays were performed in the outpatient department; the results of the blood analysis, urine analysis, amylase test and lipase test were normal. The patient had experienced a duodenal ulcer 20 years prior. There was a history of acute epigastric pain due to acute cholecystitis and gallbladder stone during the previous year. The pain was relieved by anti-inflammation treatment, and no surgery was performed.

Physical examination revealed mild tenderness in the epigastric area. Succession splash and Murphy's sign were negative. Bowel sounds were normal. Gastroscopy was arranged for the following day and indicated that chyme could be observed in the gastric antrum and duodenal bulb. A large yellowish-brown hard stone was observed in the duodenal bulb, and a 1.2-cm-wide ulcer was located on the greater curvature side of the bulb. The diagnosis of gastric retention, gastrolithiasis and duodenal bulb ulcer was considered based on the medical history and gastroscopy results. The symptoms, including abdominal distension, nausea and vomiting, were quickly alleviated following a therapy of liquid diet, antacid, gastric mucosa protectant and supportive treatment and litholytic therapy with oral sodium bicarbonate solution.

However, on the third day after admission, computed tomography (CT) of the abdomen revealed a thick gallbladder wall and the presence of gas shadows in the gallbladder. The choledoch was slightly wider, and the walls of the gallbladder and the pylorus were indistinct from each other. Additionally, a calcified mass of approximately 2 cm in diameter was observed in the pylorus. A comparison to the CT scan from one year prior (Figure 1A) revealed an inflammatory perforation of the gallblad-

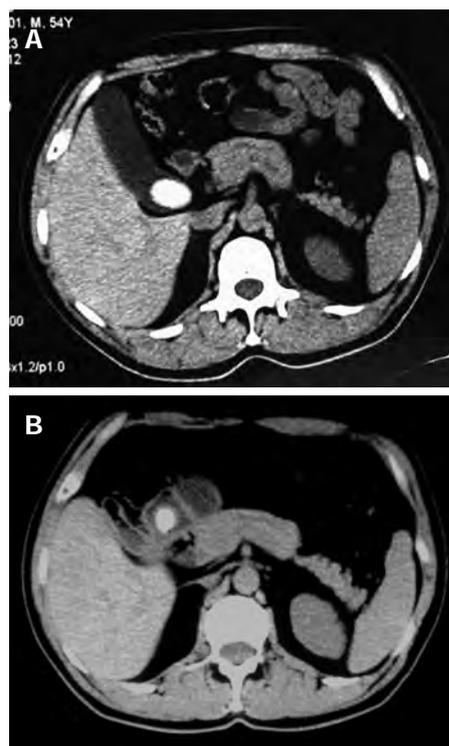


Figure 1 Abdominal computed tomography. A: Revealing a large calcified mass in the gallbladder (one year prior); B: Indicating the migration of the large stone from the gallbladder to the pylorus and gas shadows seen in the gallbladder (this time).

der and pylorus, the presence of an internal fistula and the migration of the gallstone from the gallbladder to the pylorus (Figure 1B). The upper gastrointestinal contrast also revealed a large round filling defect with a smooth border in the descending part of duodenum. Therefore, a revised diagnosis of Bouveret's syndrome was made. The patient was transferred to the laparoscopic ward and underwent duodenotomy for gallstone removal, duodenal fistulation and subtotal cholecystectomy using a laparoscope. During the operation, multiple adhesions among the gallbladder, duodenal bulb and omentum were observed. After the adhesions were released, the gallbladder-duodenal fistula could be seen. A large stone with a diameter of approximately 3.0 cm × 4.0 cm was located below the fistula-duodenal opening. After making an incision in the gallbladder wall, it was observed that the cystic duct and sinus crossings had closed. Subsequently, duodenum incision, stone removal, placement of a drainage tube into the duodenum incision and interrupted suture were performed to close the duodenum. A subtotal cholecystectomy was also performed simultaneously. After post-operative hemostatic and anti-inflammatory treatment, the patient was discharged. Three weeks later, the patient was readmitted to remove the duodenal drainage tube. On the next day after the removal, fever and abdominal pain were observed. However, an abdominal CT revealed no obvious abnormalities. After a 5-d controlled diet and anti-inflammatory treatment, the patient recovered and was discharged without further incident.

## DISCUSSION

A gastric outlet obstruction caused by the migration of large gallstones to the pylorus and duodenum *via* a gallbladder-duodenal fistula, Bouveret's syndrome, is a special type of gallstone-induced ileus that comprises only 1%-3% of cases<sup>[2,4]</sup>. This syndrome is extremely rare and appears predominantly in older women, at an average age of 74.1 years<sup>[2]</sup>. Most of these patients experience complications, such as hypertension, diabetes, chronic obstructive pulmonary disease or other systemic diseases. Bouveret's syndrome presents with the common symptoms of abdominal distension; abdominal pain; nausea; vomiting; fever; and occasionally gastrointestinal hemorrhage, such as hematemesis or melena<sup>[5,6]</sup>. Abdominal CT, upper gastrointestinal imaging and endoscopy offer important diagnostic value for diagnosing the disease<sup>[7,8]</sup>. In this case, the patient was a middle-aged male, rather than a member of the susceptible population, who presented the main manifestations, including abdominal distension, nausea, vomiting undigested food and a past history of duodenal bulb ulcer. As a result, his condition was easily misdiagnosed as duodenal bulb ulcer and pyloric obstruction. After a foreign body was observed in the duodenal bulb through endoscopy, gastrolithiasis was suspected. However, several doubts remained. First, there was no clear predisposition for gastrolith. Second, the foreign body was too large to have passed through the pylorus to arrive at the duodenum. Based on these doubts surrounding the diagnosis, the patient was submitted to further examination during litholytic and acid suppression therapy. The diagnosis was suddenly clearer based on the results of an abdominal CT. Compared with a CT image of the patient taken one year prior, it was apparent that the original large gallstone near the neck of the gallbladder had disappeared, and the gallbladder had shrunk with some gas remaining in it and a gallstone shadow in the pyloric cavity next to the gallbladder. These abdominal CT results suggested that the endoscopic finding was not a gastrolith but rather that the gallstone had penetrated into the duodenal bulb. Consequently, Bouveret's syndrome was diagnosed. Upper gastrointestinal imaging results further supported the diagnosis. The treatment choice for Bouveret's syndrome remains debatable and can include endoscopic treatment, extracorporeal shock-wave lithotripsy, intracorporeal electrohydraulic lithotripsy, surgery and laparoscopic treatment<sup>[9]</sup>. Currently, 1-stage or 2-stage surgery is often adopted<sup>[10-12]</sup>. One-stage surgery refers to a combination of enterolithotomy plus cholecystectomy and fistula repair within a single surgery. Two-stage surgery entails dividing the enterolithotomy and cholecystectomy into two operations. However, surgery is often associated with significant postoperative complications and mortality<sup>[3,13]</sup>. With the development of laparoscopic and other minimally invasive techniques, the successful application of laparoscopic treatment as a therapy for Bouveret's syndrome has also been reported<sup>[14,15]</sup>. In this case, subtotal cholecystectomy, duodenotomy for gallstone removal and duodenal fistulation

using a laparoscope were performed, taking into account the large size of the stones, the good general condition of the patient, the closed gallbladder and the duodenal fistula. The patient recovered and was discharged quickly. After the duodenal drainage tube was unplugged, no duodenal fistula was observed, and the condition of the patient remained stable after being followed up for 6 mo. The present case included the following features: (1) The patient was middle-aged, had no chronic systemic disease and did not belong to the usual susceptible population of Bouveret's syndrome; (2) The clinical symptoms were atypical, with neither infection nor abdominal pain. The onset was similar to that of peptic ulcer and pyloric obstruction, which was initially suspected as gastrolithiasis; (3) Imaging data from one year prior provided an important basis to observe the evolution of the disease and make a diagnosis; and (4) Based on the condition of the patient, disposable laparoscopic lithotomy and cholecystectomy were conducted. The effect was satisfactory, and there were no obvious postoperative complications.

In conclusion, Bouveret's syndrome is a rare cause of gastric outflow obstruction and is easily misdiagnosed. Therefore, when a patient presents an outbreak of symptoms that are similar to pyloric obstruction with a previous history of gallbladder stones, the clinician should consider Bouveret's syndrome, which can be differentially diagnosed through a combination of endoscopy and abdominal imaging. As laparoscopic technology gradually matures, the advantages of the laparoscopic treatment of this syndrome have been realized in recent years. Laparoscopic enterolithotomy is safe and effective, with good patient tolerability, rapid postoperative recovery and few wound-related complications.

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